Frozen Fertility

The challenges of ovarian tissue cryopreservation in children and adolescents with Turner Syndrome

Myra Schleedoorn

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For reasons of consistency within this thesis, some terms have been standardized throughout the text. As a consequence, the text may differ from the articles that have been published.

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Think globally, act locally

Patrick Geddes

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Introduction, aim and outline of this thesis

Chapter 1 – General introduction

"I've told her that she can't have kids, but she won't accept that. 'When I grow up I'm going to have a boy and a girl.'"
- Parent of a young female with Turner syndrome [1]

"Every once in a while, you know, when you're holding a kid and they're snuggling up to your neck, I really thought: I wish I could have kids. I wish I had the choice."
- 31-year-old female with Turner syndrome [1]

"Women get flowers and they get honoured because they're a mother, but I don't–you know, I feel, does that make them a complete woman because they were able to have children? And I couldn't, so I must be incomplete."
- 40-year-old female with Turner syndrome [1]

Turner syndrome (TS) is characterised by the partial or complete absence of a second X chromosome in some cell lines (i.e. mosaicism) or all cells. In most females, this results in early menopause and infertility. Infertility is the most painful challenge endured by females with TS, regardless of their age [1, 2]. Adolescent patients mentioned being extremely upset and disappointed at the time of diagnosis, and adult patients felt deprived of an important part of womanhood and reproductive choice [1]. The inability to bear a biological child remained, for many, a burden throughout their life, even though the majority of adult females with TS embraced alternative options to become a parent such as adoption [1].

Genotype

Typically, humans are born with 46 chromosomes arranged in 23 pairs. The first 22 pairs of chromosomes are called the autosomes and the 23rd pair includes the sex chromosomes 'X' and 'Y'. The X and Y chromosome are called sex chromosomes because they determine the person 's biological sex. Most women have a 46, XX chromosome pattern and most men 46, XY. Divergence from the normal number of X and Y chromosomes is called sex chromosome aneuploidy (SCA). One of the most common SCA in humans is TS with a birth frequency of approximately 1 in 2,500 live-born girls [3-5].
Genotypes associated with TS and their prevalence are listed in **Table 1**. Approximately 40-50 percent of females with TS have a 45,X (monosomy X or "classic TS") genotype [6]. The X chromosome is derived from the mother in two-thirds of patients and from the father in the remaining one-third [7]. This means that either an X or a Y chromosome is lost during early foetal life.

Table 1. Genotypes associated with Turner syndrome and their prevalence.

Genotype	Percent of females with TS (%)	Description
45, X	40 to 50	Monosomy X
45, X / 46, XX	15 to 25	46, XX Mosaicism
45, X / 47, XXX; **45, X / 46, XX/ 47, XXX**	3	Mosaicism with "Triple X"
45, X /46, XY	10 to 12	Mixed gonadal dysgenesis
46, XX, del(p22.3)	10 to 12	Deletion Xp22.3
46, X, r (X) / 46, XX	10 to 12	Ring X chromosome
46, X I (Xq); **46, X, idic (Xp)**	(~10)	Isochromosome Xq; Isodicentric Xp
X-autosome translocation, unbalanced	Rare	Various
46, XX, del(q24)	Rare	Not TS; premature ovarian insuffiency
46, X, idic (X)(q24)	Rare	Not TS; isodicentric Xq24

45, X with one or more other cell lines (45, X mosaicism) is found in approximately one-half of all patients with TS (e.g., 45, X / 46, XX). 45, X mosaicism results from sex chromosome nondisjunction occurring during the foetal development [8, 9]. The degree of mosaicism may differ among different tissues. Furthermore, the absence of 46, XX mosaicism in a peripheral blood sample does not always rule out the presence of 46, XX mosaicism in other tissues.

In a small percentage of TS patients, a cell line with Y chromosomal content can be identified. The presence of Y chromosomal content is of medical importance because it might result in virilisation and increased gonadal tumour risk. In these patients, prophylactic gonadectomy is recommended.

The remaining group of females with TS has a structural anomaly of the X chromosome. Examples are isochromosome Xq (46, X, I (X) q), ring chromosome X (rX) and a deletion of a portion of the short arm of the X chromosome [del(X)p].

Phenotype

A reduction of the X chromosomal content may affect the foetal development of almost all organs, leading to numerous clinical manifestations [10]. The clinical manifestations vary among girls with TS [11], and seem to be largely depending on the percentage of normal 46, XX cells, as patients with a 'full-blown' 45, X karyotype generally have a more severe phenotype [10].
The most common phenotypic features of TS are short stature and gonadal dysfunction. Other clinical features include cardio-aortic malformations, renal anomalies, hearing problems, and dysmorphic signs such as neck webbing, cubitus valgus and lymphoedema [11]. Although neurocognitive problems are common, females with TS generally have a normal range of cognitive abilities and intelligence [2]. Some of them will experience learning difficulties at school, particularly related to visual-spatial abilities, motor coordination, and mathematics [1]. Many adult women with TS are well-educated, employed, and are living a normal social life with stable relationships [2]. However, more often than females in the general population, females with TS are experiencing problems with social interaction or intimate relationships, mostly based on their concerns of being accepted despite their diagnosis [1].

Diagnosis

The majority of girls receives the diagnosis TS before the age of 5 because of growth retardation [11]. In some cases, TS is diagnosed prenatally, e.g. in case of cystic hygroma or intra-uterine growth retardation, or postnatally, e.g. in case of dysmorphic signs or congenital cardiac malformations. Occasionally, the diagnosis TS is concluded in adolescence because of delayed puberty or primary/secondary amenorrhoea.
In routine care, the diagnosis of TS is based on the genetic analysis of 20-30 peripheral blood lymphocytes [12]. On indication, the chromosome evaluation is expanded with FISH analysis on buccal cells. For instance, to identify a Y chromosome cell line in order to prevent the occurrence of a gonadoblastoma, and discuss prophylactic gonadectomy. However, new insights recommend to perform buccal cell FISH in all patients with TS [13], to obtain a better global chromosomal evaluation, and, thus better care and follow-up.

History

Prior to the availability of karyotyping, TS was reported as a clinical syndrome. The American endocrinologist Henri Turner published a case series in 1938, where he described the occurrence of short stature, sexual immaturity, neck webbing, and deformity of the

elbows (cubitus valgus) in seven independent cases [14]. A few years earlier, the German paediatrician Otto Ulrich described an eight-year-old girl with a similar phenotype [2].

Gonadal function

Females with TS are at risk of premature ovarian insufficiency and have minimal chances of spontaneous conception. In addition, the likelihood of healthy offspring in females with TS is decreased as compared to the general population, because of an increased risk for miscarriage [15], and a slightly higher chance of giving birth to a child with a congenital disorder [15, 16]. Recent studies have shown that spontaneous pregnancies in females with TS are rare and occur in 2.0 – 7.6% of patients [15, 17-20]. As TS is related with an increased risk of foetal and maternal complications [11], strict monitoring by a team of high-care obstetricians, cardiologists and anaesthesiologists is paramount during pregnancy.

Approximately one-third of females with TS will have a sufficient ovarian reserve to experience a spontaneous onset of puberty, and up to 16.1% will experience one or more spontaneous menstruation cycles [21-23]. A recent study reports even higher rates for the spontaneous onset of puberty and menstruation in females with TS [24]. Nevertheless, most females with TS will be confronted with primary or secondary amenorrhea before reaching adulthood. If these females have a desire for parenthood in the future, they are depending on assisted reproductive technologies (ART), such as oocyte donation, or alternative options to become a parent such as adoption or fostering. However, interview studies with females with TS and their parents show a strong need for new options, such as fertility preservation (FP) [1, 25].

Fertility preservation

Fertility preservation includes the cryopreservation of the patient's own gametes, either by preserving mature oocytes or ovarian tissue containing primordial follicles. Cryopreservation of mature oocytes (OC) is is an established fertility preservation approach but can be performed only in post pubertal females, as it requires ovarian activity and psychological maturity. This means that OC would be limited to a small percentage of females with TS only, i.e. girls with a spontaneous onset of menstruation who are emotionally mature enough to undergo the procedure.

The second technique, ovarian tissue cryopreservation (OTC), has the disadvantage that it requires surgery, most preferably a laparoscopic ovarian biopsy, ovarian wedge resection, or a unilateral ovariectomy. However, OTC can be performed regardless of the patient's age or pubertal development, and thus appears to be a promising technique

to preserve the fertility of young girls with TS [25]. It provides the possibility to store (some of the) primordial follicles within the ovarian tissue before their premature disappearance.
The efficacy of OTC in other patient groups has been well described, and over the past decades, several guidelines have been developed. Retransplantation of cryopreserved-thawed ovarian cortical tissue in these patients has resulted in restoration of ovarian function [26, 27], pregnancies, and healthy offspring [28-33].
Recently, a Swedish study group proved that primordial follicles can be found in the laparoscopic ovarian biopsies from young girls with TS [34]. However, if OTC in females with TS will also lead to healthy offspring is currently still unknown. Hence, exploring the feasibility and efficacy of OTC in females with TS seems a logical next step [35].
Ideally, a clinical trial should be enrolled to explore the feasibility and efficacy of OTC in young females with TS. However, caution should be exercised, because this research will involve young children and OTC requires laparoscopic surgery under general anaesthesia with a possible risk of complications [36].

Aims and outline of this thesis

The aim of this thesis was to explore the various challenges of OTC in young females with TS. This thesis serves as groundwork for an international prospective cohort study (TurnerFertility trial, CCMO NL57738.000.16, ClinicalTrial ID NCT03381300). The following research questions will be addressed in this thesis:

*I. What is currently known about the presence of follicles in the ovaries of young females with TS? (**Chapter 2: In the literature**)*

*II. What is current state of art regarding (experimental) FP outcomes in females with TS? (**Chapter 2: In the literature**)*

*III. Can we optimize the efficacy of OTC by using a non-invasive imaging technique to determine the density of follicles in human ovarian cortex fragments that are intended for fertility restoration? (**Chapter 3: Laboratory aspect**)*

*IV. Which ethical aspects should be considered regarding OTC in young females with TS? (**Chapter 4: Ethical aspect**)*

*V. What is the standpoint of an international expert panel regarding OTC in young females with TS? (**Chapter 4: Ethical aspect**)*

VI. *Which research should be performed to better inform TS patients about the feasibility and efficacy of OTC? (**Chapter 5: Clinical aspect**)*

VII. *Is it possible to determine the X chromosomal content of various ovarian cells in young patients with TS? (**Chapter 6: Genetic aspect**)*

VIII. *What is the X chromosomal content of the ovarian cells in young patients with TS? (**Chapter 6: Genetic aspect**)*

IX. *Is the X chromosomal content of the ovarian cells in young females with TS correlated with the X chromosomal content of other cell types? (**Chapter 6: Genetic aspect**)*

X. *Is FP also feasible in patients with 45, X monosomy? (**Chapter 7: Patient-specific aspect**)*

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2

In the literature

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To Freeze or Not to Freeze?
An Update on Fertility Preservation In Females With Turner Syndrome.

Pediatric Endocrinology Reviews. 2019;16(3):369-382

Abstract

Introduction

Infertility is a major concern for females with Turner syndrome (TS), regardless of their age. While fertility preservation is now routinely offered to girls and young women with cancer, there are currently no recommendations on fertility preservation in girls and young women with TS who generally face an even higher risk for infertility. Despite the lack of international guidelines, preservation procedures have been performed experimentally in females with TS.

Methods

A systematic literature search based on the PRISMA-P methodology for systematic reviews was performed in order to collect all published data on fertility preservation options in females with TS between January 1980 and April 2018. A total number of 67 records were included in this review. The records were screened for information regarding cryopreservation of mature oocytes and ovarian tissue in females with TS. Two ongoing trials on fertility preservation in young females with TS were also included.

Results

Cryopreservation of oocytes or ovarian tissue has been performed experimentally in > 150 girls and adolescents with TS over the last 16 years. The efficacy of fertility preservation options in females with TS is still unknown due to the lack of follow-up data.

Conclusion

The efficacy of fertility preservation procedures in females with TS is still unknown. Future studies with focus on efficacy, safety and long-term follow-up are desperately needed.

Key words

Turner syndrome – Fertility preservation - Premature ovarian insufficiency – Ovarian tissue cryopreservation – Cryopreservation of oocytes

Introduction

Turner syndrome (TS) is a condition in which a female is partly or completely missing one sex chromosome. It is the most common chromosomal abnormality in females, affecting 1 on 2,500 live-born girls [1-3]. The complete or partial loss of one sex chromosome in females leads to dysmorphic features and a constellation of physical findings that often includes short stature, cardiac and renal abnormalities, and infertility. Intelligence is generally normal among females with TS, but neurocognitive and psychosocial sequelae are common.

For many years, it was thought that girls with TS were born infertile due to non-functional ovaries. However, scientists questioned this theory as increasing numbers of spontaneous pregnancies in females with TS were described. In 2002, a Swedish study group reported that primordial follicles were found in the ovaries of 8 out of 9 patients with TS who underwent a laparoscopic ovarian biopsy [4]. However, the number of follicles found was significantly lower as compared to the follicular density in unaffected girls undergoing laparoscopic ovarian biopsy. This new insight led to the hypothesis that a complete or partial loss of one sex chromosome in females causes an accelerated degeneration of germ cells, starting at the 13th week of fetal age [5-7]. In the general population, females are born with an ovarian reserve of approximately 1-2 million oocytes. This number is reduced to approximately 300.000 at puberty and continues to decrease throughout life until menopause is reached [8]. Due to the rapid loss of the ovarian reserve, most females with TS reach menopause during childhood or adolescence. The timeline at which this occurs is less clear, and may be different for each individual patient with TS [6]. Recent studies have shown that spontaneous pregnancies in females with TS are rare and occur in 2.0 – 7.6% of patients [9-13]. Approximately one-third of females with TS will have a sufficient ovarian reserve to experience a spontaneous onset of puberty, and up to 16.1% will experience one or more spontaneous menstruation cycles [8, 14, 15]. A recent study reports even higher rates for the spontaneous onset of puberty and menstruation in females with TS [16]. However, the majority of females with TS will be confronted with primary or secondary amenorrhea before reaching adulthood.

Dealing with infertility can be one of life's most stressful experiences, and interview studies [17, 18] have shown that, regardless of age, this is one of the main concerns of females with TS and their parents. For this reason, it is recommended that girls with TS are informed early during childhood or adolescence about the possibilities to fulfil their wish for a future child. An expectative management approach can be proposed, especially in females with regular menstruation cycles. However, the majority of females with TS currently depend on assisted reproductive technologies (ART), such as oocyte donation, or alternative options to become a parent such as adoption or fostering. Quantitative and qualitative research shows a strong need for new options such as fertility preservation [17, 19].

Fertility preservation includes the cryopreservation of the patient's own gametes, either by preserving mature oocytes or ovarian tissue containing primordial follicles. In recent years, thanks to the success rates in other patient groups [20-23], physicians are often asked by girls with TS and their parents about the options to preserve their fertility [24]. However, very little is known about the success rates of different fertility preservation options in this specific group of patients. This raises the question if the promise of fertility preservation is at present hypothetical, or if these options can be realistically offered to females with TS.

Methods

A systematic literature search based on the PRISMA-P methodology for systematic reviews [25] was performed in Pubmed in April, 2018 in order to collect all published data on fertility preservation in females with TS. A total of 2021 records could be identified by using search terms mentioned in **Appendix I**. The search was limited to studies published in English or Dutch between January 1980 and April 2018 with full text availability. This resulted in a total number of 1276 articles. The selection of relevant articles was based on title and abstract screening, which was done by four investigators. In case of doubt, the titles and abstracts were discussed with the other investigators. Additional articles were found by scanning the reference list of each relevant article. A total number of 73 articles were screened in full text by two investigators independently, which led to the exclusion of 26 articles. Reasons for exclusion were publication in other languages than Dutch or English, no full text availability, or being irrelevant to the main topic. Additionally, 20 useful publications were included after the screening of reference lists. A total number of 67 records were included in this review and were screened for information regarding fertility preservation in females with TS **(Figure 1)**. Information was gathered on both cryopreservation of mature oocytes and ovarian tissue. Two ongoing trials on fertility preservation in young females with TS were also included. Information regarding embryo freezing in patients with TS was not taken into account, as this review focuses on fertility preservation in females with TS without a male partner.

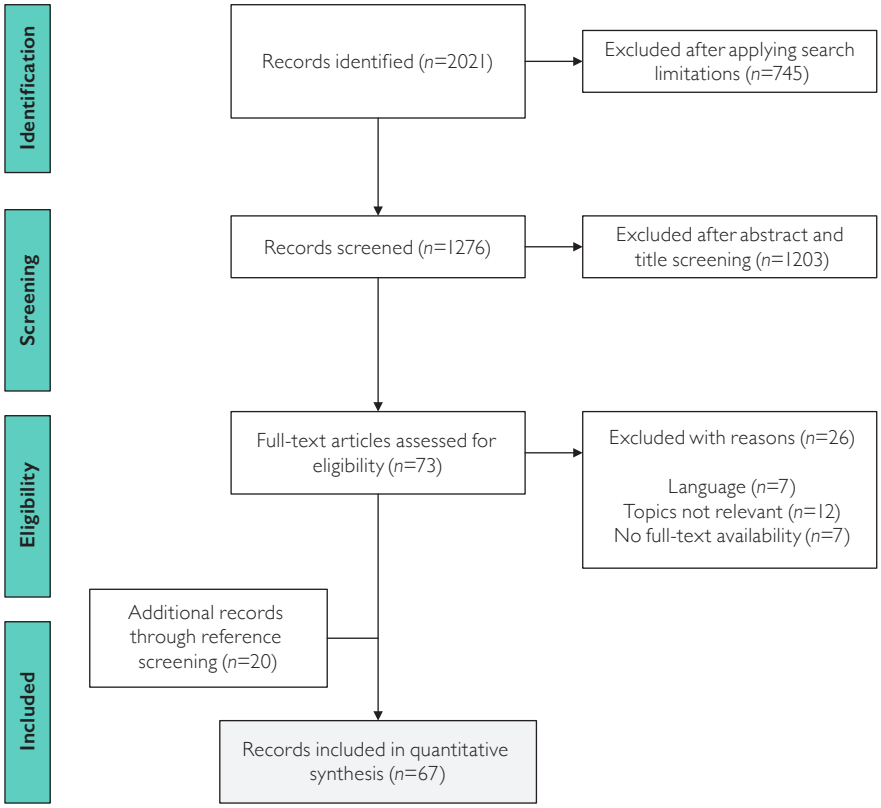

Figure 1. Flow diagram of the search process

Results

Cryopreservation of mature oocytes in females with TS

Cryopreservation of mature oocytes (OC) requires ovarian stimulation with exogenous FSH administration followed by transvaginal ultrasound-guided oocyte retrieval [26]. OC is an established fertility preservation approach but can be performed only in post pubertal females, as it requires ovarian activity and psychological maturity. This means that OC is limited to a small percentage of females with TS, namely those who will experience a spontaneous onset of menstruation and are emotionally mature enough to undergo the procedure.

Over the last ten years, eighteen cases of OC in females with TS have been reported [27-34]. Patient characteristics and biochemical profiles are summarized in **Table 1.**

All eighteen cases experienced a spontaneous onset of menstruation. Fifteen females had a 45, X / 46, XX karyotype with a 45, X percentage between 20 – 93%, two females had a 45, X / 47, XXX karyotype with a 45, X percentage between 90 – 98%, and one female was diagnosed with 45, X (86%) / 46, XX (3%) / 47, XXX (11%) TS. In four patients OC was opted because of an irregular cycle. Thirteen females still had a regular menstruation cycle when they decided for OC. In one patient, the regularity of the menstruation cycle was not reported. The age of patients during the oocyte retrieval ranged from 13 to 28 years. Before ovarian stimulation, eight patients had a normal FSH serum level, and ten patients had a serum FSH level of >15 mUI/mL. Serum AMH levels ranged from 1.7 to 43.8 pmol/L. Ultrasonic assessment showed an antral follicle count between 6 and 40, with a mean number of 13 antral follicles. The stimulation medication, dosage, type of trigger and OC outcomes are detailed in **Table 1**. On average, 11 oocytes (range 2 – 19) were retrieved per cycle, of which approximately 76% could be cryopreserved (range 69 – 100%). All females tolerated the procedure well, and in most females OC was performed safely without any complications. However, one patient developed a severe ovarian hyper stimulation syndrome. Seven days after the administration of 5.000 IU human chorionic gonadotropin (hCG), she had to undergo an abdominal paracentesis of 1L of ascites, which resulted in a full recovery. The peak estradiol level on the day of the trigger in this patient was high (16.545 pmol/L) compared to the peak estradiol levels that was described in the other cases (range 1.241 – 5.956 pmol/L.

There are no published reports of pregnancies after OC in patients with TS.

Ovarian tissue cryopreservation in females with TS

Ovarian tissue cryopreservation (OTC) requires a surgical procedure, most preferably a laparoscopic ovarian biopsy, ovarian wedge resection, or a unilateral ovariectomy. OTC can be performed regardless of age or spontaneous onset of puberty, and thus appears to be a promising technique to preserve the fertility of young girls with TS [19]. It provides the possibility to store a part of the primordial follicles within the ovarian tissue before their premature disappearance. Even though OTC in girls facing fertility threatening cancer treatment is accepted and established [16-18], there are currently no recommendations on fertility preservation in girls with TS. However, OTC has been performed experimentally in at least 83 young females with TS aged 6 – 19 years old [4, 19, 28, 31, 35]. Study characteristics and outcomes are summarized in **Table 2**. Ovarian tissue could be obtained in approximately 85% of the patients, and no complications related to the surgical procedure have been reported. Primordial follicles were found in the ovarian tissue of 43% of the cases. Positive predictive markers for the presence of follicles were mosaic TS, a spontaneous menarche, a spontaneous thelarche, a normal FSH level, and a normal AMH level. One study reported a positive association with age (above 12 years old) [19], which might be related to the age distribution within

the study group, as 86% of the study population was 12 years or older. None of the other studies reported a correlation with the patient's age. The follicular density in the ovarian cortex tissue harvested in young females with TS varied from 0 – 1200 follicles per mm^3. It is unknown if autotransplantation of earlier cryopreserved ovarian tissue has already been performed in patients with TS. There has to date been no follow-up data published.

Currently, the data from 47 girls with TS who underwent OTC in France is being assessed and will be reported soon (personal communication Lise Duranteau). One clinical trial currently in progress in the Netherlands, is recruiting young females with TS aged 2 to 18 years (principal investigator Kathrin Fleischer). This cohort will be followed from OTC until live birth with the aim to collect long-term follow-up data (**Table 3**).

Table 1. Cryopreservation of mature oocytes in patients with Turner syndrome - reported cases.

Authors	**Kavoussi et al 2008**	**Huang et al 2008**	**Lau et al 2009**	**El-Shawarby et al 2010**
Country	United States	Canada	Canada	United Kingdom
Procedure(s)	Cryopreservation of mature oocytes	Cryopreservation of mature oocytes and ovarian tissue	Cryopreservation of mature oocytes	Cryopreservation of mature oocytes
η	1	1	1	1
Age (y)	28	16	16	22
Karyotype	45, X / 46, XX (percentages not reported)	45, X (20%) / 46, XX (80%)	45, X (98%) / 47, XXX (2%)	45, X (86%) / 47, XXX (11%) / 46, XX (3%)
Cells used for chromosome analysis	not reported	lymphocytes (number not reported)	not reported	not reported
Spontaneous thelarche	yes	yes	yes	yes
Spontaneous menarche	yes	yes	yes	yes
Menstruation cycle	irregular	irregular	regular	regular
Serum AMH	not reported	not reported	not reported	8.5 pmol/L
Serum FSH	4.3 mIU/ml	9.8 mUI/mL	6.3 mUI/mL	4.6 mUI/mL
Serum LH	not reported	1.4 mUI/mL	2.4 mUI/mL	not reported
Serum estradiol	not reported	119 pmol/L	73.4 pmol/L	<44.0 pmol/L
Serum inhibin B	not reported	not reported	not reported	not reported
Oocyte retrieval	Ovarian hyper stimulation followed by transvaginal oocyte retrieval under ultrasonographic guidance	Laparoscopic wedge resection of the left ovary and in vitro aspiration of the antral follicles, followed by the isolation of (immature) oocytes and in vitro maturation from germinal vesicle stage to metaphase II stage.	Ovarian hyper stimulation followed by transvaginal oocyte retrieval under ultrasonographic guidance	Ovarian hyper stimulation followed by transvaginal oocyte retrieval under ultrasonographic guidance
Antral follicle count	40	not reported	6	7
№ of cycles	1	not applicable	1	1
Protocol	antagonist	not applicable	agonist	flare-up (agonist)

Balen et al 2010	**Oktay et al 2010***	**Oktay et al 2014***			**Balkenende et al 2015**
United Kingdom	United States	United States			The Netherlands
Cryopreservation of mature oocytes and ovarian tissue	Cryopreservation of mature oocytes	Cryopreservation of mature oocytes			Cryopreservation of mature oocytes
1	1	3			10
28	14*	13	14*	13	not reported
45, X (93%) / 46, XX (7%)	45, X (45%) / 46, XX (55%)	45, X (90%) / 47, XXX (10%)	45, X (45%) / 46, XX (55%)	45, X (20%) / 46, XX (80%)	45, X / 46, XX (100%)
30 lymphocytes	20 lymphocytes	30 lymphocytes	20 lymphocytes	20 lymphocytes	not reported
yes	yes	yes	yes	yes	not reported
yes	yes	yes	yes	yes	not reported
regular	irregular	irregular	irregular	not reported	regular
43.8 pmol/L	2.0 pmol/L	3.6 pmol/L	2.0/3.8 pmol/L **	1.7 pmol/L	not reported
not reported	5.3 mUI/mL	5.7 mUI/mL	5.3 mUI/mL	5.6 mUI/mL	> 15.0 mUI/mL
not reported	9.5mUI/mL	3.9 mUI/mL	9.5 mUI/mL	5.3 mUI/mL	not reported
not reported	239.3 pmol/L	33.9 pmol/L	146.5 pmol/L	75.3 pmol/L	not reported
106.2 pg./mL	<30.0 pg/mL	54.8 pg/mL	<30.0 pg/mL	47.2 pg/mL	not reported
Ovarian hyper stimulation followed by transvaginal oocyte retrieval under ultrasonographic guidance	Ovarian hyper stimulation followed by transvaginal oocyte retrieval under general anesthesia and ultrasonographic guidance	Ovarian hyper stimulation followed by transvaginal oocyte retrieval under general anesthesia and ultrasonographic guidance	Ovarian hyper stimulation followed by transvaginal oocyte retrieval under general anesthesia and ultrasonographic guidance	Ovarian hyper stimulation followed by transvaginal oocyte retrieval under general anesthesia and ultrasonographic guidance	Ovarian hyper stimulation followed by transvaginal oocyte retrieval under ultrasonographic guidance
not reported	12	6	12	6	not reported
2	1	1	2	1	not reported
antagonist	antagonist	antagonist	antagonist	antagonist	not reported

Table 1. Continued.

Authors	Kavoussi et al 2008	Huang et al 2008	Lau et al 2009	El-Shawarby et al 2010
Stimulation medication and dose	2.025 IU rFSH (sd 1-9)	not applicable	900 IU rFSH (sd 1-10) + 150 IU hMG (sd 6-10)	825 IU hMG (sd 1-11)
Duration of ovarian stimulation (days)	9	not applicable	10	11
Peak estradiol levels on the day of trigger	16.545pmol/L	not applicable	1.241 pmol/L	5.956 pmol/L
Trigger medication and dose	5.000 IU hCG	not applicable	hCG, dose not reported	10.000 IU hCG
Number of oocytes retrieved per cycle	15	not applicable	2	8
Number of mature oocytes cryopreserved	13	8	2	8
Total number of mature oocytes cryopreserved	13	8	2	8
Complications	Ovarian hyperstimulation syndrome	none	none	none

** One of the three cases included in the publication of Oktay et al 2014 was already reported by the same study group as a case report in 2010. ** AMH levels were measured prior to the first stimulation and prior to the second stimulation. *** Oocytes retrieved in the first stimulation cycle were used for FISH analysis.*

Balen et al 2010	**Oktay et al 2010***	**Oktay et al 2014***			**Balkenende et al 2015**
1.350 IU rFSH (sd 1-9)	1.800 IU rFSH (sd 1-10), 150 IU hMG (sd 8-10)	2.475 IU rFSH + 150 IU rLH (sd 1-11)	1.800 IU rFSH + 450 hMG (sd 1-10) / 3.750 IU rFSH + 2.100 hMG (sd 1-14)	2.025 IU hFSH + 75 IU rLH (sd 1-10)	not reported
9	10	11	10 / 14	10	not reported
not reported	5.112pmol/L	3.478 pmol/L	5.112/4.559 pmol/L	3.624 pmol/L	not reported
10.000 IU hCG	1 mg luprolide acetate	3300 IU hCG	1 mg luprolide acetate / 1 mg luprolide acetate	250mcg rhCG	not reported
12*** / 10	11	19	11 / 7	16	not reported
0*** / 10	8	10	8 / 4	12	not reported
10	8	10	12	12	not reported
none	none	none	none	none	not reported

Table 2. Cryopreservation of ovarian tissue in patients with Turner syndrome - reported cases.

Authors	**Hreinsson et al 2002**	**Huang et al 2008**	**Borgström et al 2009**
Country	Sweden	Canada	Sweden
Procedure(s)	Cryopreservation of ovarian tissue	Cryopreservation of mature oocytes and ovarian tissue	Cryopreservation of ovarian tissue
Surgical intervention	Laparoscopic removal of a quarter to one whole ovary	Laparoscopic ovarian wedge resection	Laparoscopic unilateral ovarian biopsies (25-50% of the ovarian cortical tissue of one of both ovaries)
Study period	not specified	not specified	not specified
η	10	1	57
Age (y)	12 – 19	16	8-19
Karyotype (% of participants)	45, X / 46, XX (50%),45, X (40%), and 45, X / 46, XX / 47, XXX (10%)	45, X / 46, XX (100%)	45, X (49%), structural X chromosomal abnormalities (38%), 45, X / 46, XX (9%), 45, X / 46, XX / 47, XXX (2%), 45, X / 47, XXX (2%)
Cells used for chromosome analysis	lymphocytes or skin fibroblasts (number not reported)	lymphocytes (number not reported)	15-105 lymphocytes (no case specific reports)
Ovarian tissue could be obtained (% of participants)	9 (90%)	1 (100%)	47 (82%)
Complications related to the surgical procedure	not reported	not reported	none
Presence of follicles in the obtained ovarian tissue (% of participants)	8/9 (89%)	1/1 (100%)	15/47 (32%)
Positive predictive markers for the presence of follicles (positive predictive value, statistical significance)	small sample size	small sample size	Mosaic Turner syndrome (0.86, p = 0.0001), spontaneous menarches (0.62, p = 0.0039), spontaneous thelarche (0.58, p = 0.0008), normal FSH level (0.69, p = 0.0123), normal AMH level (0.64, p = 0.0003), age 12-16 years old (0.31, not significant), age above 16 years old (0.24, not significant)

Balen et al 2010	**Von Wolff et al 2015**	**Jadoul et al 2017**	**Jensen et al 2017**
United Kingdom	Germany, Switzerland, Austria	Belgium	Denmark
Cryopreservation of mature oocytes and ovarian tissue	Cryopreservation of mature oocytes or ovarian tissue (not specified)	Cryopreservation of ovarian tissue	Cryopreservation of ovarian tissue
Laparoscopic bilateral ovarian biopsies	not specified	Laparoscopic ovarian biopsies	Laparoscopic unilateral salpingo-oophorectomy
1998 - 2009	2007-2013	1997-2013	2000-2015
1	8	not reported	6
17	not reported	not reported	6-17
45, X / 46, XX (100%)	45, X mosaicism (100%)	not reported	45, X (67%), 45, X mosaicism (33%)
30 lymphocytes	not reported	not reported	not reported
1 (100%)	not reported	not reported	6 (100%)
none	not reported	not reported	not reported
1/1 (100%)	not reported	not reported	not reported
small sample size	not reported	not reported	not reported

Table 2. Continued.

Authors	Hreinsson et al 2002	Huang et al 2008	Borgström et al 2009
Negative predictive markers for the presence of follicles (negative predictive value, statistical significance)	small sample size	small sample size	45, X karyotype (0.89, p = 0.0001), structural X chromosomal abnormalities (0.73, p = 0.0001), no spontaneous menarche (0.81, p = 0.0039), no spontaneous thelarche (0.87, p = 0.0008), elevated FSH level (0.77, p = 0.0123), low AMH level (0.88, p = 0.0003), age below 12 years old (0.82, not significant)
Follicles per mm³ in ovarian cortex	0-499	not reported	0-1200
Follow up data	not reported	not reported	not reported
Optimal age to discuss ovarian tissue cryopreservation	All patients with TS, ideally before the age of 12-13 years old, especially in females with nonmosaic Turner syndrome	All patients with TS, ideally aged 12-13 years old	All patients with TS, ideally aged 13-14 years old

Balen et al 2010	Von Wolff et al 2015	Jadoul et al 2017	Jensen et al 2017
small sample size	not reported	not reported	not reported
not reported	not reported	not reported	not reported
not reported	not reported	not reported	not reported
not reported	not reported	not reported	not reported

Table 3. Cryopreservation of ovarian tissue in patients with Turner syndrome - ongoing trials.

Authors	**Duranteau et al (ongoing trial)**	**Fleischer et al (ongoing trial)**
Country	**FR**	**NL**
Procedure(s)	Cryopreservation of ovarian tissue	Cryopreservation of ovarian tissue
Surgical intervention	Laparoscopic unilateral ovariectomy	Laparoscopic unilateral ovariectomy
Start date	January 2011	January 2018
Estimated inclusion completion date	December 2015	January 2021
Estimated end of study date	February 2031	January 2021 (proximate) January 2071 (primary outcome measure)
Primary outcome measure	Ovarian follicular density	Live birth rate (LBR) after autotransplantation of cryopreserved-thawed ovarian cortical tissue Proximate: the number of primordial follicles found in the ovarian tissue
Secondary outcomes measures	Hormonal markers (FSH, AMH, Inhibin B and estradiol) before, at one month and one year after ovariectomy	• The association between patient's age at cryopreservation and LBR • The association between patient's genotype and LBR • The association between patient's AMH level at cryopreservation and LBR • The association between patient's FSH level at cryopreservation and LBR
Other outcome measures	None	• The willingness of females with Turner syndrome to perform a unilateral ovariectomy for fertility preservation (i.e. the study participation rate) • The number of eligible participants • The age of the participant • The incidence of somatic mosaicism (i.e. buccal cells versus peripheral lymphocytes) • The incidence of germ cell mosaicism (i.e. oocytes versus peripheral lymphocytes and buccal cells) • Serum hormone levels (i.e. FSH, LH, AMH, E2, inhibin B) • The number of complications related to the laparoscopic procedure

		• The incidence of spontaneous puberty and/or spontaneous menarche after laparoscopic oophorectomy • The incidence of spontaneous pregnancies after laparoscopic oophorectomy • The incidence of menstruation cycle recovery after autotransplantation of cryopreserved-thawed ovarian tissue in the future • The incidence of pregnancies after autotransplantation of cryopreserved-thawed ovarian tissue in the future • The number of ongoing pregnancies after autotransplantation of cryopreserved-thawed ovarian tissue in the future • The number of miscarriages after autotransplantation of cryopreserved-thawed ovarian tissue in the future • Time to pregnancy after autotransplantation of cryopreserved-thawed ovarian tissue in the future • Time to live birth after autotransplantation of cryopreserved-thawed ovarian tissue in the future
η	47 (not recruiting)	38/100 (active, still recruiting)
Inclusion criteria	• Girls and young females diagnosed with Turner syndrome or mosaic Turner aged 1 through 25 years, • Without any severe (cardiovascular) co morbidity, • With ovarian insufficiency dated < 5 years in patients older than 18 years old • With signed informed consent by the patient and/or her parents	• Girls and young females diagnosed with Turner syndrome, mosaic Turner (e.g. 45, X/46, XX) or Turner variants (e.g. isochromosome X) aged 2 through 18 years, • Who completed the diagnostic work up phase of Turner syndrome including routine cardiac screening, • With signed informed consent by the patient and/or her parents
Exclusion criteria	• Girls and young females diagnosed with Turner syndrome or mosaic Turner with contra indications to perform any surgery, and/or • With a single ovary presence, and/or • HIV, HBV, HCV and/or syphilis TPHA VDRL infection, and/or • Without health insurance coverage	• Girls and young females diagnosed with Turner syndrome, mosaic Turner or Turner variants with contra indications to perform a laparoscopic unilateral oophorectomy under general anesthesia (e.g. severe cardiovascular co morbidity and/or BMI >40 kg/m2), and/or • Contra-indications to perform cryopreservation (i.e. an active HIV, hepatitis-B or hepatitis-C infection)
Cells used for chromosome analysis	not reported	a minimum of 30 lymphocytes and 100 buccal cells. In some patients, additional chromosome analysis of urine and ovarian cells will be performed
NCT Number (ICMJE)	NCT01410045	NCT03381300

Discussion

Cryopreservation of mature oocytes and ovarian cortex tissue has been performed experimentally in > 150 girls and adolescents with TS [4, 19, 27-37] [Clinicaltrial.org]. Thus far, there are no published records of girls with TS who have returned for auto-transplantation or *in vitro* fertilization with embryo transfer. Recently, the success rate of cryopreservation and subsequent transplantation(s) of ovarian tissue in cancer survivors was reported [23]. The FertiPROTEKT study group found a restoration of ovarian function in 67% per transplantation. Pregnancy and live birth rates were 33 and 25% per transplantation, respectively [23], and multiple autotransplantation sessions per patient were possible, depending on the number of ovarian strips. However, these data cannot be automatically translated to females with TS, as the occurrence of pregnancy and live birth after autotransplantation of frozen ovarian cortex strips is highly correlated with the number of functional primordial follicles found in the ovarian tissue [38]. Women with TS are likely to have a decreased follicular density [19, 39], but also show a higher risk of miscarriage [11], and a slightly higher risk of a child with a congenital disorder when they conceive spontaneously [11, 40]. If these increased risks are related to the functional integrity or the chromosome profile of their follicular cells remains unclear. Autotransplantation of ovarian cortex fragments with a low number of functional follicles might lead to false hope, as it is unlikely that these cortex strips will lead to restoration of ovarian function, pregnancy and live birth. Possibly, *in vitro* activation (IVA) of residual dormant follicles [41-44] prior to autotransplantation can be applied to optimize the treatment outcome. However, no cases of IVA in patients with TS have been reported so far.

The possible negative effects on future fertility of girls with TS after the removal of one ovary are also unknown. Recent studies in other patient groups have shown that the surgical removal of one ovary does not affect the menstrual cycle, nor the chances of getting pregnant in the future [45-48]. However, removal of an ovary may lead to an earlier onset of menopause of up to 3 years in comparison to females who still have both ovaries [49]. In these patient groups it has been suggested that one intact ovary may compensate for the loss of the other [50].

Hence, fertility restoration by OC or OTC in patients with TS is at present still hypothetical, and further research is needed to provide supporting evidence for the efficacy of these fertility preservation techniques in this specific group of patients. The Oncofertility Consortium states that OTC is a promising approach to provide fertility options to girls with TS [51]. In some countries, OTC is already routinely offered to girls with TS.

It is fundamental that young females with TS and their parents are not only informed about the chances of developing POI and the possibilities to preserve their fertility, but also receive information on alternative options to become a future parent (e.g. by

oocyte donation, surrogacy, adoption or fostering). Furthermore, they should be counselled about the probability of conceiving spontaneously, even if the occurrence of spontaneous pregnancies is low (2.0 – 7.6%). In addition, the maternal and fetal risks of a future pregnancy should be discussed, including extensive counselling about the cardiovascular risks. Needless to say, it is preferable to discuss fertility preservation options with girls who are competent to make their own decisions. However, the majority of girls are infertile by the end of adolescence. This young age of reaching infertility may be a limiting factor in comprehension and emotional maturity required to fully understand their fertility status and preservation options. The International Turner Consensus Group therefore states that fertility preservation counselling should begin at the time of diagnosis to allow the patient and her parent(s) to have enough time to consider its implications. However, they recommend against routine fertility preservation of young TS girls before the age of 12 years [52].

A decision aid based on the information needs of girls with TS and their parents is therefore required to facilitate deliberate decision making. Ideally, this decision aid should include a patient-specific *a priori* calculation of the ovarian reserve, with the aim to avoid unnecessary interventions (i.e. in girls without follicles in their ovaries or in females with a normal ovarian reserve) and to better pinpoint the urgency of fertility preservation. Several studies have been conducted aiming to estimate the ovarian reserve in patients with TS at a young age [19, 26]. A spontaneous onset of puberty and menstruation, mosaic karyotype, measurable AMH level, and normal FSH level have been associated with the presence of follicles [19, 26].

Borgström et al. suggested the following categories of girls to have the highest chance of having follicles: 1) girls with a mosaic karyotype; 2) girls with a spontaneous onset of puberty and 45, X or 45, X / 46, X and structural anomalies; and 3) girls with normal serum FSH and/or normal AMH levels with or without spontaneous onset of puberty. By retrospectively applying these criteria to their cohort [19], 19 laparoscopic procedures would have been performed, and 23 girls would have been excluded from taking an ovarian biopsy. In 15 girls the surgical procedure would have been postponed. No follicles would have been found in 8 out of 19 girls, and 2 girls with follicles would have been missed, resulting in a relatively low sensitivity of 0.58 and a specificity of 0.91. Using these criteria for fertility preservation counselling in females with TS is still controversial, as there are, for example, also spontaneous pregnancies reported in females with a 45, X monosomy karyotype (classical TS) [10-12, 53-59]. Nevertheless, it should be questioned whether these females indeed had a true 45, X karyotype and not a low grade 45, X / 46, XX mosaicism karyotype, as the number of cells used for chromosome analysis are often not reported.

Thus, optimal discriminative markers to estimate the ovarian reserve in females with TS are still lacking. For this reason, and because of its experimental character, experts recommend against routine fertility preservation in females with TS [19, 26, 52, 60]

Future clinical research should focus on both the efficacy of fertility preservation options in females with TS including pregnancy rates, pregnancy outcomes, and maternal, as well fetal risks, and on the development of a reliable prognostic model for the presence of follicles in females with TS.
In vitro studies with the aim to optimize the chances for females with TS of becoming biological parents of their own children should focus on oocyte genetics, activation of immature oocytes in isolated ovarian tissue [41-44], maturation of immature oocytes obtained from cryopreserved ovarian tissue (IVM) [61], and the development of artificial gametes from stem cells [62].

Conclusion

Cryopreservation of oocytes or ovarian tissue has been performed experimentally in > 150 girls and adolescents with TS since 2002. However, the efficacy of fertility preservation procedures in females with TS is still unknown. Future studies with focus on efficacy, safety and long-term follow-up are desperately needed.

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Appendix 1. Search terms

((Ullrich Turner Syndrome* [tiab] OR Turner Syndrome* [tiab] OR Turner's Syndrome* [tiab] OR Turners Syndrome* [tiab] OR Gonadal Dysgenesis, 45,X [tiab] OR Gonadal Dysgenesis, XO [tiab] OR XO Gonadal Dysgenesis [tiab] OR Monosomy X [tiab] OR Bonnevie-Ullrich Syndrome [tiab] OR Status Bonnevie-Ullrich [tiab] OR TS [tiab] OR "Turner Syndrome*"[Mesh]) AND ("Fertility Preservation"[Mesh] OR preservation[tiab] OR Fecundability [tiab] OR Fecundity [tiab] OR Subfecundity [tiab] OR Fertility [tiab] OR Infertility [tiab] OR subfertility [tiab] OR sub-fertility [tiab] OR fertile [tiab] OR pregnan* [tiab] OR infertile [tiab] OR ovarian insufficiency [tiab] OR Ovarian Failure [tiab] OR ovarian dysfunction [tiab] OR ovary insufficiency [tiab] OR ovary failure [tiab] OR ovary dysfunction [tiab] OR "Fertility "[Mesh] OR "Pregnancy"[Mesh] OR "Primary Ovarian Insufficiency"[Mesh]))

3

Laboratory aspect

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Assessment of reflectance confocal microscopy for non-invasive selection of optimal ovarian cortex fragments for autotransplantation.

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Abstract

Research question
Can reflectance confocal microscopy (RCM) be used to determine follicle density in human ovarian cortex fragments that are intended for fertility restoration?

Design
RCM was used on living cortex tissue fragments derived from five bovine ovaries and 13 human ovaries. All tissue fragments were cryopreserved and thawed before RCM analysis. Follicle numbers and distribution were determined by RCM and histology. Before and after RCM, general tissue viability and follicle integrity were assessed by a glucose uptake assay and neutral red staining, respectively.

Results
RCM can detect all stages of follicle development in living ovarian tissue to a maximum depth of 250 µm. In bovine tissue, all follicles were located within this 0–250 µm range. In human ovarian tissue, follicles were also present below the 250 µm RCM threshold, implying that only a percentage of the total number of follicles could be detected with RCM. The percentage of follicles detected by RCM appeared to be age dependent. The RCM procedure did not affect the glucose uptake by the tissue, whereas neutral red staining indicated a high level of follicle survival.

Conclusion
In this proof of concept study, we have shown that RCM is a promising technique to determine the density of follicles ex vivo in living human ovarian cortex fragments, apparently without compromising the vitality of the tissue. Safety studies and further optimization of the RCM technique with a focus on increasing the penetration depth are required before clinical use of RCM.

Key message
Reflectance confocal microscopy (RCM) is a promising technique to visually determine the density of follicles ex vivo in living human ovarian cortex fragments without compromising the vitality of the tissue. Safety studies and further optimization of the RCM technique are required before RCM can be used in a clinical setting.

Introduction

Since the first successful autotransplantation of frozen-thawed ovarian tissue was reported 14 years ago [1], ovarian tissue cryopreservation (OTC) has become an established fertility preservation option for selected groups of patients, as for example patients undergoing gonadotoxic treatments [2-4]. After the patient has been cured and experiences difficulties to conceive, one or more cryopreserved tissue fragments can be thawed and transplanted back into the patient in an attempt to restore her fertility.

In Europe, OTC is routinely performed in 12 countries with an estimated number of 2500-6500 procedures per year [5]. Several national studies have reported that autotransplantation of frozen-thawed ovarian tissue leads to restoration of ovarian function in 67-93% of cases [4, 6-8] with reported live birth rates between 25-33% per transplantation [4, 7, 9-12]. In a study with a limited number of patients a live birth rate of 75% has been reported [8]. To date, more than 130 children have been born following autotransplantation of cryopreserved ovarian tissue [3, 4, 7, 13-15].

Depending on the size of the ovary, the method used to obtain the ovarian cortex fragment(s), and the patient's age, 11-30 ovarian cortex fragments are prepared for cryopreservation [16]. Restoration of ovarian function in the future is most likely related to the number of primordial follicles in the autograft [6, 17]. Preferably, the cortex fragments with the highest number of primordial follicles should be selected for autotransplantation purposes. In addition, the number of cortex fragments transferred during a transplantation procedure might be adjusted if the number of primordial follicles present in the individual cortex fragments was known in advance.

Determining the follicular density with conventional histology renders the tissue unsuitable for subsequent transplantation. In addition, follicles are not evenly distributed, and therefore determining follicle density in one tissue fragment does not necessarily reflect the number of follicles in adjacent fragments [18, 19]. Ideally, a non-invasive imaging technique is required to determine the number of follicles in each ovarian cortex fragment prior to autotransplantation.

Optical coherence tomography (OCT) has been reported as a promising technique for the non-invasive imaging of ovarian tissue at the cellular level [20-23], but unfortunately its detection range in ovarian tissue is limited to a maximum depth of 100 µm. Alternatively, reflectance confocal microscopy (RCM) [24] could be used to evaluate the number of primordial follicles in ovarian cortex tissue fragments. RCM is a non-invasive imaging technique that has thus far been used mainly in dermatology for the *in vivo* detection of skin cancer [25]. RCM provides *en face*, high-resolution images and has the potential to cover the complete surface of a standard ovarian cortex tissue fragment (40-50 mm^2) in minutes. Furthermore, RCM produces images in skin up to a depth of 250 µm with a resolution similar to conventional light microscopy [26]. RCM

has never been used to analyse human ovarian tissue. Therefore, as a proof of concept, we developed an *ex vivo* method for imaging ovarian cortex fragments. In addition, the influence of RCM imaging procedure on tissue viability was evaluated.

Materials and methods

Study design

Reflectance confocal microscopy (RCM) was performed using the commercially available and CE-marked Vivascope® 1500 (*Lucid Inc., Rochester, BY, USA*) imaging system (**Figure 1**). This RCM system uses a diode laser with a near-infrared wavelength of 830nm and a tissue penetration up to 200-300 µm in skin [27]. RCM images were compared with histological images of the same tissue fragments obtained with conventional microscopy of haematoxylin and eosin (HE) stained sections. HE stained tissue sections images were digitized using the VisionTek® (*Sakura Finetek Europe B.V, Alphen a.d. Rijn, NL*) live digital microscope. The distribution and total number of follicles, the follicular density and other histological aspects of the ovarian tissue were assessed in both bovine and human ovarian cortical tissue fragments. In addition, the effect of RCM imaging on ovarian tissue viability and follicle viability was evaluated. Lastly, the percentage of primordial follicles within the detection range of RCM was determined.

Ovarian tissue preparation, cryopreservation and thawing

Intact bovine ovaries of 7-8 months old animals were collected at a local abattoir and transported to the laboratory on ice, essentially as described previously [28]. Bovine ovarian tissue has been proposed as the best animal model to study human ovarian tissue [29] .

Human ovarian tissue was collected from 13 patients between January 2014 and December 2017 after ethical approval (Ethical committee Arnhem and Nijmegen, reference numbers: 2016-2894 and 2016-2871, date of approval: 12 October 2016 and 19 December 2016; Ethical committee Amsterdam, reference number: 2017.168, date of approval: 28 March 2017; and Ethical committee Bonn reference number: 007/09, date of approval: 11 May 2008) was obtained for patient-related research (**Table 1**). 9 patients (aged 5 to 40 years) underwent unilateral ovariectomy for fertility preservation purposes at the University Hospital of Bonn or at the Radboud University Medical centre. 4 patients (aged 18 to 27 years) underwent bilateral ovariectomy as part of their gender affirming surgery at the VU Medical centre Amsterdam. In accordance with the Declaration of Helsinki, all 13 patients and/or their parents had provided written informed consent for the use of their ovarian tissue for research purposes. Ovarian tissue was prepared for cryopreservation and thawing as previously described [30]. Cortex fragments of approximately 8x6x1mm were used for RCM imaging.

Figure 1. The Vivascope 1500 system. Before imaging, the ovarian cortex tissue fragment was transferred to the specially designed pre-cooled adaptor for ex-vivo imaging (arrowhead) and covered by a sterile plastic window and a magnetic metal ring. Ultrasound gel was applied to the window and the objective lens housing was attached to the metal ring. The system allows real-time visualization of living tissue and generates black and white images on the computer screen that are stored digitally.

Qualitative analysis – Comparing RCM images of ovarian cortex fragments with the corresponding HE stained sections

Since the Vivascope® 1500 was initially designed for *in vivo* imaging of the skin only, a special sample container was designed (**Figure 1**) to perform *ex vivo* RCM imaging of ovarian tissue. The sample container was sterilized prior to every use. Ovarian cortex fragments of standard size (8x6x1mm) could be easily fitted in this sample holder. Furthermore, the viability of the ovarian fragment that was examined could be safeguarded, as each ovarian tissue fragment was moistened by a sponge soaked in cold L15 medium during imaging. The sample holder was placed in a second container filled with ice to ensure that the tissue remained at 0°C during the imaging procedure. The moistened ovarian tissue fragment was covered by an optical plastic window that was subsequently stuck to the steel ring of the sample container. Before imaging, ultrasound gel was applied to the centre of the optical window in order to generate the confocal image. The laser head was positioned on the steel ring with a magnetic snap.

Table 1. Characteristics of patients from whom ovarian cortex tissue was obtained.

Patient characteristics	**Human samples (*n*=13)**
Mean age (median age; SD, range)	20,77 years *(21,00 years; 9,24 years, range 5-40 years)*
Age group, n (%)	
Paediatric (0-11 years)	1 *(7,69%)*
Adolescent (12-17 years)	4 *(30,77%)*
Young adult (18-29)	6 *(46,15%)*
Adult (>30 years)	2 *(15,38%)*
Indication for OTC, n (%)	
Oncologic	7 *(53,85%)*
Rhabdomyosarcoma	1 *(7,69%)*
Cervical cancer	2 *(15,38%)*
Breast cancer	1 *(7,69%)*
Osteosarcoma	1 *(7,69%)*
Acute myeloid leukaemia	1 *(7,69%)*
Hodgkin lymphoma	1 *(7,69%)*
Gender dysphoria	4 *(30,77%)*
Other	2 *(15,38%)*
Bone marrow transplantation for hyper eosinophilic syndrome (HES)	1 *(7,69%)*
Metachromatic leukodystrophy	1 *(7,69%)*

The RCM examination was systematically performed starting from the tunica albuginea down to the medulla. Horizontal black-and-white images of 8x8mm (Vivablock) (covering the complete surface of the tissue fragment) with an axial resolution of 5.0 µm and a lateral resolution of 0.5 - 1.0 µm close to optical histology, were obtained by capturing a series of confocal images of 500x500 µm with vertical steps of 25 µm using the Vivascan Version 7 software (Lucid Inc., Rochester, BY, USA). RCM images were constructed based on the specific reflectance index of the single tissue structure due to its molecular properties.

After the RCM imaging procedure was completed, the tissue samples were fixed in Bouin solution and embedded in paraffin for conventional histological analysis. The RCM images were then compared with the corresponding horizontal HE stained 6 µm sections by two investigators. Small spots of Indian ink were used to properly align the RCM images to the corresponding HE stained sections.

Viability assays – Determining the viability of ovarian tissue and ovarian follicles using glucose uptake assay and Neutral red staining

The viability of ovarian cortical tissue after RCM imaging

To assess the possible adverse effects of RCM imaging on the quality of the ovarian cortical tissue fragments, the viability of the tissue fragments before and after RCM imaging was determined by an *in vitro* glucose uptake assay as previously described [20, 31]. Frozen-thawed bovine cortical ovarian tissue fragments of 5 individual animals were exposed to a complete RCM analysis for the duration of 30 minutes at 0 °C. Frozen-thawed bovine cortical ovarian tissue fragments of the same ovaries that were not imaged, served as a control and were kept on ice for 0, 30, 60 or 120 minutes. The tissue fragments were cut into small (approximately 1x1x1 mm) pieces that were subsequently cultured for 3 days in 2 ml Dulbecco's Modified Eagle's medium (DMEM) culture medium supplemented with 10% Foetal Calf Serum (FCS) and 40 µg/ml gentamycin. Glucose content of the culture supernatant was determined and compared with fresh culture medium. The weight of each cultured tissue sample was determined at the end of culture. Glucose consumption was expressed as nmol glucose per milligram of ovarian cortical tissue per hour. Bovine ovarian tissue of two animals, which was snap-frozen and thawed in the absence of cryoprotectants, served as a positive control for tissue damage.

The viability of the primordial follicles after RCM imaging

The viability of the primordial follicles before and after the RCM imaging was determined by a Neutral red staining assay as previously described, [20, 31, 32]. Neutral red staining is a commonly used assay to determine the viability of primordial follicles *in vitro.* The number of viable (red-coloured) follicles and non-viable (non-coloured) follicles was calculated by using standard light microscopy. Frozen-thawed bovine cortical ovarian tissue fragments of 5 individual animals were exposed to RCM for 30 minutes. Frozen-thawed bovine cortical ovarian tissue fragments of the same animals that were not subjected to imaging, served as a control.

Quantitative analysis – Determining the percentage of primordial follicles within the detection range of RCM

Frozen-thawed ovarian tissue samples from 5 animals and all 13 patients (**Table 1**) were fixed with Bouin solution and embedded in paraffin for conventional histological analysis. Follicles were categorized according to predefined criteria [33]. Vertical HE stained sections were used to create side-view images for an overview of the localization of the primordial follicles throughout the ovarian cortex and more specifically, within the detection range of RCM. The number of primordial follicles from the tunica albuginea down to the medulla was determined by two investigators independently. Afterwards, the total number of primordial follicles at different depths was calculated

for several ranges: 0 - 50 µm, 50 - 100 µm, 100 - 150 µm, 150 - 200 µm, 200 - 250 µm and deeper than 250 µm.

Statistical analysis

Student t test was used to perform statistical analysis, using SPSS software (version 22.0, BM, New York). P-values of 0.05 or less were considered to be statistically significant.

Results

RCM imaging of bovine ovarian cortex fragments

In view of the paucity of human ovarian cortex tissue available for research purposes, we initially resorted to bovine tissue for the optimization of the RCM imaging procedure. Starting at the tunica albuginea, which mostly consists of extracellular matrix fibrils, each fragment was scanned down towards the medulla. Using RCM, each ovarian cortex fragment could be visualized at the cellular level in black and white images, up to a maximum depth of approximately 250 µm (**Figure 2**). Follicles could be easily distinguished from other ovarian tissue structures in bovine ovarian tissue. Whereas oocytes appeared as dark circular structures, the surrounding granulosa cells appeared as small circular or white oval structures. After passing the tunica albuginea (**Figure 2, panel B**), small (primordial and primary) follicles with a diameter of approximately 35-40 µm were abundantly present from a depth of 50 µm up to 175 µm (**Figure 2, panels C-H**). In addition to primordial follicles with a flat layer of granulosa cells, we also observed primary follicles surrounded by cuboidal granulosa cells. More advanced stages of follicular development with a diameter of 80 µm or more showed multiple layers of granulosa cells and were located at a depth of 125 µm or deeper (**Figure 2, panels F-I**). *Ex vivo* RCM imaging up to depth of 250 µm of an ovarian tissue fragment with a surface of 8x6mm took about 30 minutes.

To confirm that the RCM images of a tissue fragment corresponded with the HE staining of the same fragment, we first produced RCM images of each ovarian cortex tissue fragment. Next, several easily recognizable RCM images were selected and compared with the HE stained sections taken at the same depth from which the RCM images originated. As shown in **Figure 3** the follicles in the RCM images corresponded well to both size and location of the follicles in the HE stained sections.

To determine the percentage of small follicles in bovine ovarian cortex tissue within the detection range (0-250 µm) of RCM, transversal HE stained tissue sections were produced from ovarian tissue of 5 animals. In these sections the number of primordial/primary follicles was determined at various distances from the cortex surface. The small follicles were found to be all located within the detection range of RCM (**Figure 4**).

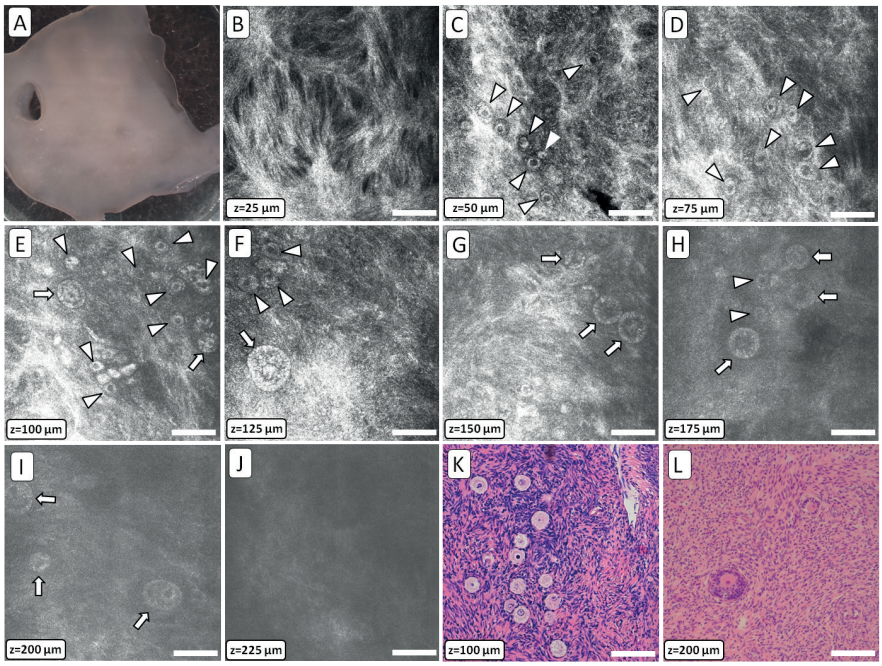

Figure 2. Reflectance confocal microscopic (RCM) images of bovine ovarian cortex tissue. (A) A cryopreserved and thawed bovine ovarian cortex fragment of approximately 8x6x 1 mm thick was imaged using RCM at different levels (z = 25–225 µm), with steps of 25 µm starting from the cortex surface (B–J). After RCM imaging, sections of the tissue were stained with haematoxylin and eosin at two different depths (K and L). Arrowheads point to small (primordial and primary) follicles, arrows point to more advanced stages of follicular development. Scale bars represent 100 µm.

Determining the viability of ovarian tissue and follicles after RCM

The RCM imaging of each ovarian cortex fragment was performed at 0^0 C within 30 minutes using a non-ionizing near-infrared laser. The possible adverse effects of the RCM imaging procedure on the viability of ovarian tissue and primordial follicles were determined by two viability assays; an *in vitro* glucose uptake assay to determine overall tissue viability and a Neutral Red stain to assess follicle viability [20, 31, 32]. **Figure 5a** shows the mean glucose consumption per mg ovarian tissue per hour over a 3-day culture period of ovarian cortex fragments from 5 animals that were exposed to 30 minutes of RCM imaging at 0°C. Glucose uptake was compared with cortex tissue of the same ovary that had not been subjected to RCM imaging but was stored at 0°C for various periods of time (0-30-60-120 minutes). As shown in **Figure 5a** for cortex tissue from 5 individual animals, glucose uptake by the tissue subjected to RCM was not

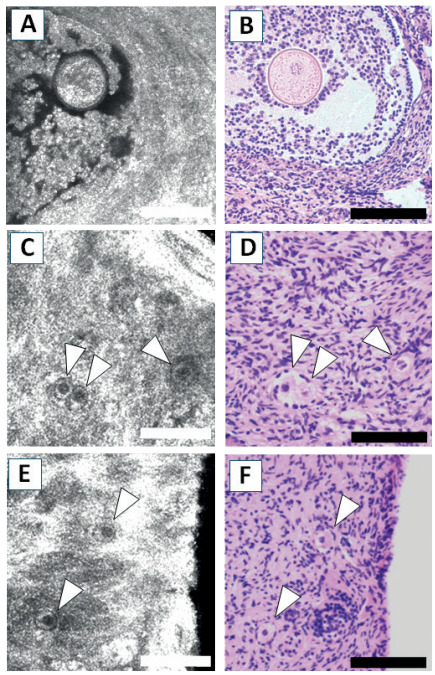

Figure 3. Reflectance confocal microscopic (RCM) images with corresponding haematoxylin and eosin stained sections. Several unique RCM images of a bovine ovarian cortex fragment were selected showing a large antral follicle (A), two closely located small follicles (C) and small follicles close to the edge of the tissue fragment (E). After RCM, a series of haematoxylin and eosin stained sections was produced. The location of the follicles with RCM corresponds highly to the location of the follicles observed in conventional haematoxylin and eosin histology (B, D and F). Arrowheads point to small (primordial and primary) follicles. Scale bars represent 100 µm.

affected by the imaging procedure and statistically not different from tissue that was stored on ice for either 0 or 30 minutes ($p > 0.05$). Even a longer incubation of 60 or 120 minutes on ice did not influence glucose uptake by the tissue (data not shown). As a positive control for tissue damage, ovarian tissue fragments of 2 animals were snap-frozen in the absence of cryoprotectants resulting in a strong reduction in glucose uptake. In addition to the overall tissue viability, the possible effect of RCM imaging on the viability of the primordial follicles within the ovarian tissue fragment was examined. To this end, small follicles in frozen-thawed bovine ovarian tissue fragments from three individual animals were stained with Neutral Red before and after a 30-minute-long RCM imaging session (**Figure 5b**). In all tissue samples, irrespective of RCM imaging, the percentage of Neutral Red stained primordial follicles was between 85-98%. This

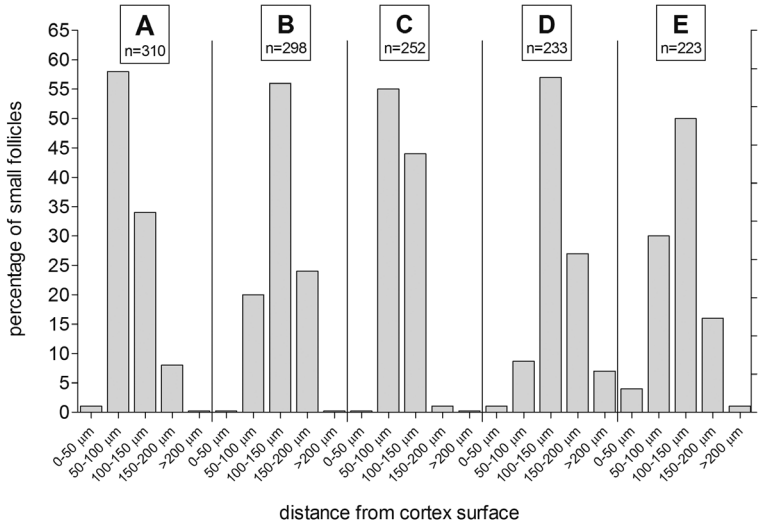

Figure 4. Vertical distribution of small follicles in bovine ovarian cortex tissue. The number of small (primordial and primary) follicles in transversal haematoxylin and eosin stained sections at different levels from the cortex surface was determined in ovarian tissue of five animals (A–E). The total number of small follicles counted for each individual animal is indicated.

indicates that RCM imaging for 30 minutes at 0°C does not affect the viability of bovine ovarian tissue nor the viability of the small follicles.

RCM imaging of human ovarian cortex fragments

RCM analysis of a cryopreserved/thawed ovarian cortex fragment of a 5-year-old patient revealed the presence of many small follicles already at a tissue depth of 25 µm (**Figure 6, panel B**). Similar to the bovine images, the follicles appeared as dark spheres surrounded by a lighter coloured ring. In this particular tissue, follicles were abundantly present up to a depth of 200 µm (**Figure 6, panels B-I**), after which the RCM resolution became too low for accurate detection of individual follicles. The follicle density observed in HE stained tissue sections was in agreement with the density we observed using RCM (**Figure 6, panels K and I**). With RCM, follicles of different sizes could also be detected in the ovarian cortex fragments of adult patients, as shown in **Figure 7** for a 22 years old patient. Up to a depth of 50 µm only a small number of follicles were seen (**Figure 7, panels A-C**), but beyond this depth patches of many small follicles could be observed that were unevenly distributed throughout the cortex (**Figure 7, panels D-H**). Interestingly, in this particular tissue, RCM was able to detect follicles beyond the 200 µm limit, allowing us to detect larger follicles (with a diameter up to 100 µm) at 200 µm or deeper.

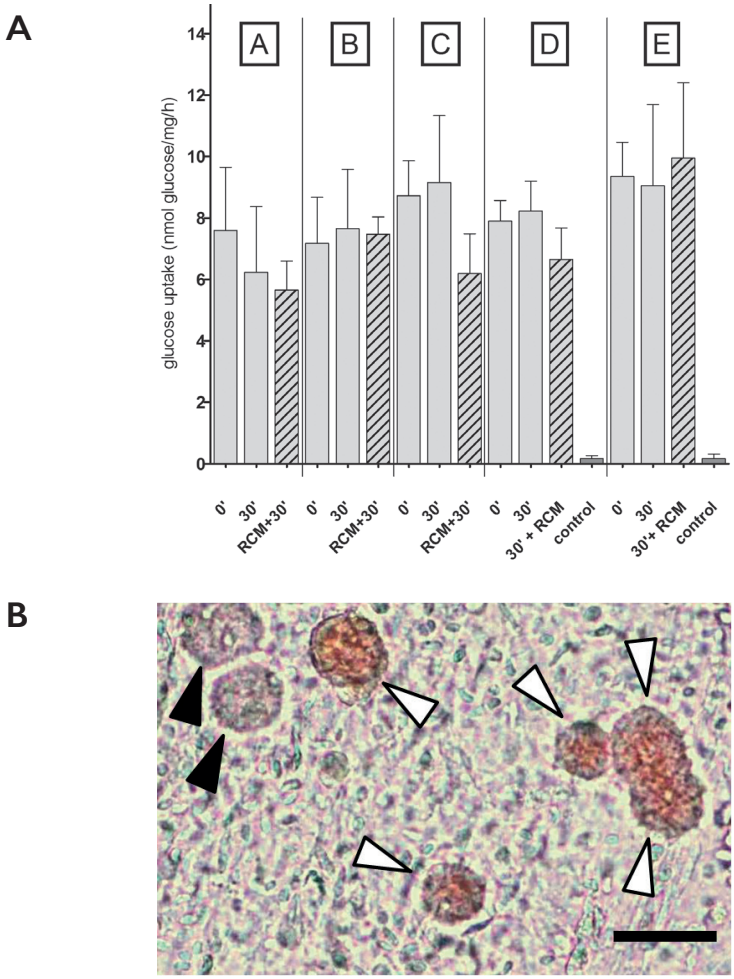

Figure 5. A) Viability of ovarian cortex fragments after reflectance confocal microscopic (RCM) imaging. In-vitro glucose uptake over a 3-day culture period of ovarian cortical tissue fragments derived from five bovine ovaries (A–E) after incubation on ice for 0 or 30 min and after tissue was subjected to 30 min of RCM imaging at 0°C (hatched bars). No statistical difference in glucose uptake between control tissue (0 or 30 min on ice) and tissue after 30 min of RCM imaging was observed ($P > 0.05$). As a positive control for tissue damage the glucose uptake was measured for ovarian tissue from two animals (D and E) that was snap-frozen–thawed in the absence of cryoprotectants, leading to a statistically significant reduction in glucose uptake ($P < 0.05$). Values presented as mean ± SD; (B) follicles visualized by neutral red staining. Overview of follicles from a bovine ovarian cortical tissue fragment subjected to 30 min of RCM imaging at 0°C stained with neutral red. Viable follicles are red (white arrowhead) and dead follicles remain transparent (black arrowheads). Scale bar represents 50 µm.

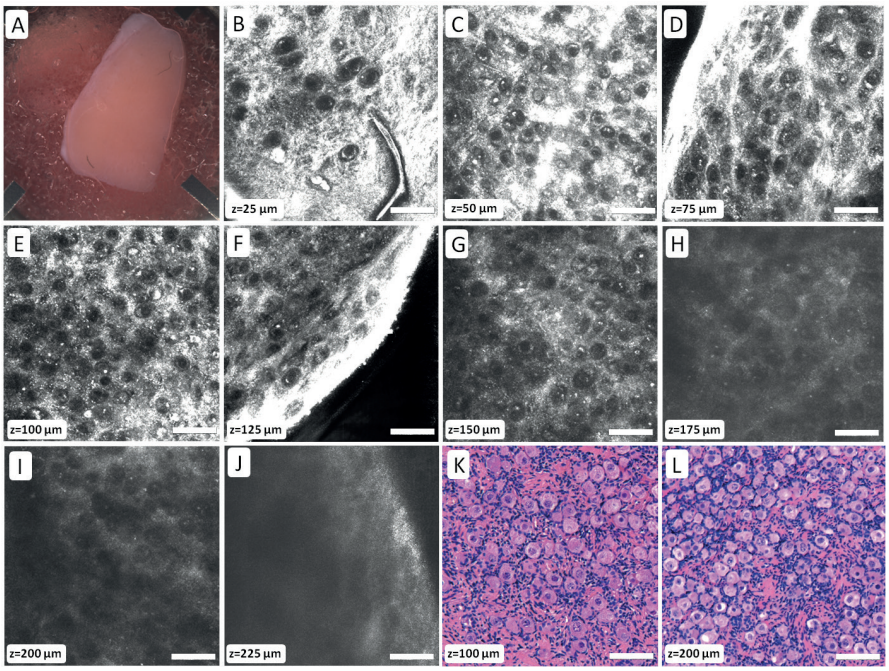

Figure 6. Reflectance confocal microscopic (RCM) images of ovarian cortex tissue from a 5-year old patient. (A) A cryopreserved and thawed human ovarian cortex fragment of approximately 8x6x1 mm thick of a 5-year old patient was imaged using RCM at different levels (z = 25–225 µm) with steps of 25 µm starting from the cortex surface (B–J). After RCM imaging, sections of the tissue were stained with haematoxylin and eosin at two different levels (K and L). Small (primordial and primary) follicles were observed in high numbers at all levels. Scale bars represent 100 µm.

To determine the percentage of small follicles that were located within the detection range of RCM in human ovarian cortex tissue, transversal HE stained sections of ovarian cortex tissue of patients of different ages were used. The number of small follicles at different depths was counted by 2 investigators independently (**Figure 8a**). We observed that the distance between the small follicles and the cortex surface increased with age. In the ovarian cortex fragments derived from 3 patients aged 5, 12 and 14 years, respectively, 65%, 14% and 47% of the follicles were located within the detection range of the RCM. In the patients aged 16 – 33 years old, 10% or less of the follicles was located within this detection range, whereas in the oldest patient (40 years of age), all remaining small follicles were located 250 µm or deeper, and therefore outside the detection range of RCM (**Figure 8b**).

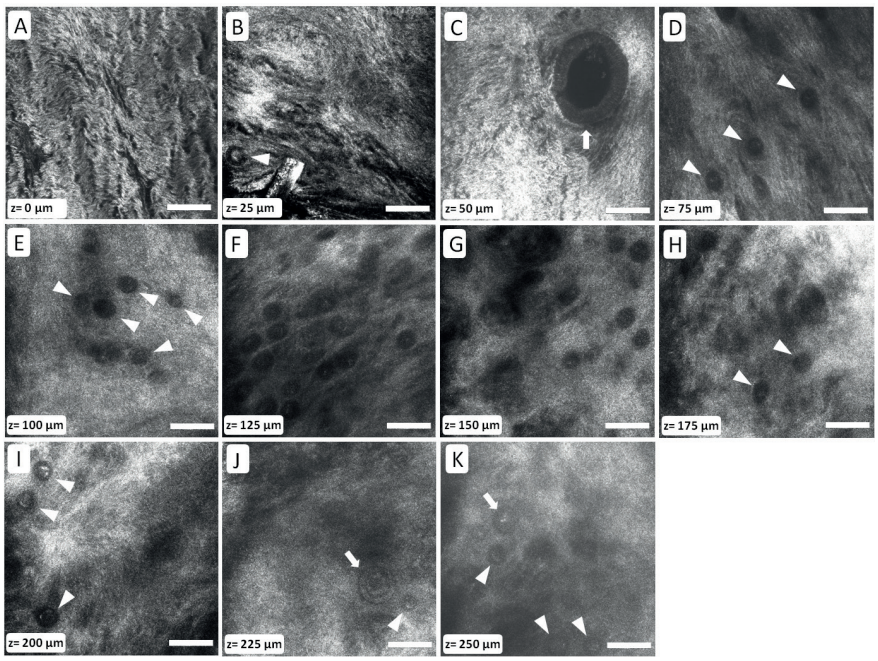

Figure 7. Reflectance confocal microscopic imaging (RCM) images of ovarian cortex tissue from a 22-year old patient. A cryopreserved and thawed human ovarian cortex fragment of approximately 8x6x1 mm thick of a 22-year old patient was imaged using RCM at different levels (z = 0–250 µm), with steps of 25 µm starting from the cortex surface (A–K). Arrowheads indicate small (primordial and primary) follicles and arrows point to more advanced stages of follicular development. Scale bars represent 100 µm.

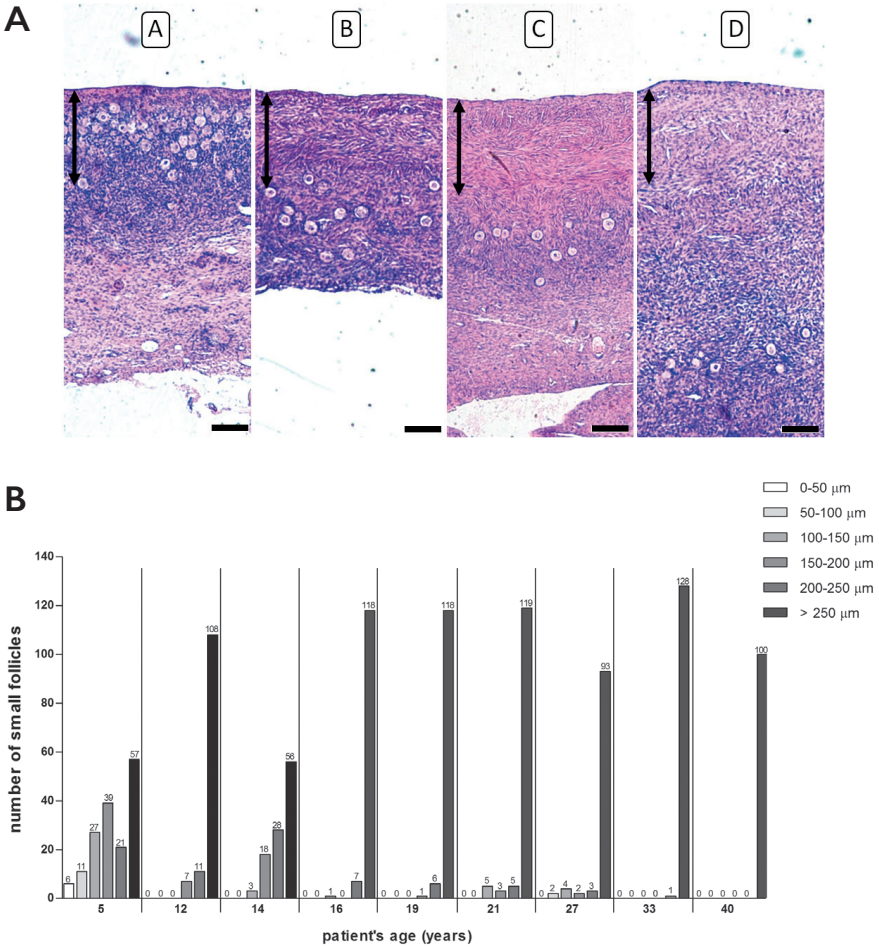

Figure 8. Vertical distribution of small (primordial and primary) follicles in human ovarian cortex in patients of different ages. (A) Transversal haematoxylin and eosin stained sections of human ovarian cortical tissue of patients aged 5 years (A), 19 years (B), 33 years (C) and 40 years (D). Note the increase in distance between the remaining small follicles and the ovarian cortex surface with advancing age. The detection range of reflectance confocal microscopic images in human ovarian cortical tissue (approximately 0–250 µm) is indicated by a double headed arrow. Scale bars represent 100 µm; (B) quantitative analysis of the vertical distribution of small follicles in human ovarian cortical tissue. Small follicles were counted in transversal haematoxylin and eosin stained sections of human ovarian cortical tissue of patients of various ages and categorized according to their distance from the ovarian cortex surface. The number of small follicles that lies within (0–50 µm, 50–100 µm, 100–150 µm, 150–200 µm and 200–250 µm), and outside (>250 µm) the detection range of RCM is indicated. Note that, with advancing age, the remaining follicles are found increasingly further away from the cortex surface.

Discussion

In this proof of concept study, we have investigated whether reflectance confocal microscopy (RCM) can be used as a non-invasive imaging technique to determine the vertical and horizontal distribution, and the number of follicles in ovarian cortical tissue fragments intended for fertility preservation purposes. Our results show that RCM could readily detect all follicles in a bovine ovarian cortex tissue fragment of approximately 45 mm^2 in 30 minutes, up to a depth of 250 µm. Ovarian tissue and follicle viability were not affected by the imaging procedure. Tissue damage was not expected as RCM uses a point light source derived from a near-infrared laser of 830 nm that delivers 5-10 mW non-ionizing radiation power [27]. Furthermore, RCM imaging was performed in a specially designed container under sterile conditions at 0^0C to avoid microbial contamination or additional ischemic damage to the tissue. Incubation of ovarian tissue at 0^0C for up to 120 minutes did not result in a significant reduction in the viability of the ovarian tissue and follicles, indicating that the 30 minutes RCM procedure had very little effect on the ovarian cortex fragment. However, more research is needed on the functional integrity of the ovarian tissue after exposure to RCM.

By comparing RCM images and HE stained sections of the same ovarian tissue fragment, we show that RCM is capable of visualizing all stages of follicle development, including primordial follicles. Remarkably, the follicle visibility was generally better in bovine tissue compared with human tissue. In bovine ovarian tissue, follicles could be easily detected up to 250 µm, which is comparable with the maximum imaging depth of RCM reported in skin tissue [25, 34-37].

In human ovarian tissue, follicles could be easily detected up to a depth of 100 µm, which is comparable to the maximum imaging depth of optical coherence tomography (OCT) reported in human ovarian tissue [20-23]. However, between 100 µm and 250 µm, follicles could still be detected, but were more difficult to distinguish from other ovarian tissue structures due to a limited absorption of laser light and scattering. This seemed to be related to the thickness of the tunica albuginea which increases with age [38]. This layer of extra cellular matrix reduces the tissue penetration of the near-infrared laser light, and hence, to a decrease in reflectance [39]. In ovarian cortex tissue from young patients, the tunica albuginea was found to be significantly thinner, and RCM was capable of detecting up to 65% of the follicle population in the tissue of our 5-year-old patient. Thus, RCM seems an appropriate non-invasive imaging method to estimate the follicular density in young patients. However, in ovarian cortex from adult patients, only a small percentage of the total number of follicles could be detected with RCM, making it difficult to determine the follicular density in these patients.

In theory, the detection range of RCM could be doubled by scanning each ovarian tissue fragment from both sides. However, the medullar side of the approximately 1 mm thick cortex fragment hardly contains any follicles in the first 250 µm and will

therefore not significantly contribute to the total number of follicles that can be observed by RCM. A further improvement of the RCM technique could increase the maximum tissue penetration depth in human ovarian tissue, and hereby increase its predictive value. After improvement, RCM could be applied to evaluate the follicular density in ovarian tissue fragments of patients who are undergoing ovarian tissue cryopreservation, and in the future, ovarian tissue fragments with the highest number of primordial follicles could be selected for autotransplantation first. This is of clinical importance in all patients undergoing ovarian tissue autotransplantation, especially in patients with a diminished ovarian reserve, as the chances of the restoration of their fertility critically depends on the number of primordial follicles in the autograft [40]. However, more research is required to confirm that the numbers of follicles observed with RCM are indeed correlated with the capacity of the tissue to restore fertility after autotransplantation.

The present study is the first to evaluate the use of RCM for the non-invasive selection of optimal ovarian cortex fragments for autotransplantation. RCM is a promising technique to visually determine the density of follicles *ex vivo* in living ovarian cortex fragments without compromising the vitality of the tissue. RCM is capable of visualizing all stages of follicle development, including primordial follicles up to a maximum depth of 250 µm, hence leaving the majority of the follicles undetected. Safety studies and further optimization of the RCM technique are required before this non-invasive imaging technique can be used in a clinical setting.

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4

Ethical aspect

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International consensus: ovarian tissue cryopreservation in young Turner syndrome patients: outcomes of an ethical Delphi study including 55 experts from 16 different countries.

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Abstract

Study question
What is the standpoint of an international expert panel on ovarian tissue cryopreservation (OTC) in young females with Turner syndrome (TS)?

Summary answer
The expert panel states that OTC should be offered to young females with TS, but under strict conditions only.

What is known already
OTC is already an option for preserving the fertility of young females at risk of iatrogenic primary ovarian insufficiency (POI). Offering OTC to females with a genetic cause of POI could be the next step. One of the most common genetic disorders related to POI is TS. Due to an early depletion of the ovarian reserve, most females with TS are confronted with infertility before reaching adulthood. However, before offering OTC as an experimental fertility preservation option to young females with TS, medical and ethical concerns need to be addressed.

Study design, size, duration
A three-round ethical Delphi study was conducted to systematically discuss whether the expected benefits exceed the expected negative consequences of OTC in young females with TS. The aim was to reach group consensus and form an international standpoint based on selected key statements. The study took place between February and December 2018.

Participants/materials, setting, methods
Anonymous panel selection was based on expertise in TS, fertility preservation or medical ethics. A mixed panel of 12 gynaecologists, 13 (paediatric) endocrinologists, 10 medical ethicists, and 20 patient representatives from 16 different countries gave consent to participate in this international Delphi study. In the first two rounds, experts were asked to rate and rank 38 statements regarding OTC in females with TS. Participants were offered the possibility to adjust their opinions after repetitive feedback. The selection of key statements was based on strict inclusion criteria.

Main results and the role of chance
A total of 46 participants completed the first Delphi-round (response rate 84%). Based on strict selection criteria, six key statements were selected, and 13 statements were discarded. The remaining 19 statements and two additional statements submitted by the expert panel were re-evaluated in the second round by 41 participants (response

rate 75%). The analysis of the second survey resulted in the inclusion of two additional key statements. After the approval of these eight key statements, the majority of the expert panel (96%) believed that OTC should be offered to young females with TS, but in a safe and controlled research setting first, with proper counselling and informed consent procedures, before offering this procedure in routine care. The remaining participants (4%) did not object but did not respond despite several reminders.

Limitations, reasons for caution

The anonymous nature of this study may have led to lack of accountability. The selection of experts was based on their willingness to participate. The fact that not all panellists took part in all rounds may have resulted in selection bias.

Wider implications of the findings

This international standpoint is the first step in the global acceptance of OTC in females with TS. Future collaborative research with a focus on efficacy and safety and long-term follow-up is urgently needed. Furthermore, we recommend an international register for fertility preservation procedures in females with TS.

Study funding/competing interest(s)

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Introduction

Removal of one of the two ovaries for cryopreservation followed by autotransplantation of ovarian cortex fragments in the future (i.e. ovarian tissue cryopreservation: OTC) is already an option for preserving the fertility of young females at risk of iatrogenic primary ovarian insufficiency (POI) [1-4]. Offering OTC to females with a genetic cause of POI could be the next step. One of the most common genetic conditions related to POI is Turner syndrome (TS), affecting 25-50 per 100,000 live-born girls [5].

TS is caused by the partial or complete absence of one of the sex chromosomes. Missing an X or Y chromosome affects the foetal development. Signs and symptoms vary greatly among females with TS but are mostly related to the patient's karyotype. Monosomy 45, X is associated with a more severe lymphatic and skeletal phenotype, while the dysmorphic features of patients with a mosaic karyotype can be very mild, depending on the level of mosaicism. No association was found between karyotype and cardio-aortic malformations [6]. The majority of females with TS are diagnosed before the age of 12 years because of growth retardation [7]. TS may be diagnosed prenatally by chorionic villus sampling or amniocentesis in cases of foetal cystic hygroma,

intra-uterine growth retardation, cardiovascular malformations or advanced maternal age, or shortly after birth because of dysmorphic signs such as neck webbing, cubitus valgus, and lymphoedema. In a few cases, TS is diagnosed in adolescence because of delayed puberty or primary or secondary amenorrhea.

Females with TS are known to have a shorter reproductive lifespan due to an accelerated loss of germ cells. This process starts during meiosis I of the foetal oocyte and continues until the point when the ovarian reserve is completely exhausted [8, 9]. In most females with TS, this point is reached during childhood or early adolescence [9]. Up to 33% have some pubertal development and 10-15% experience one or more spontaneous menstruation cycles [10-12]. Hence, spontaneous pregnancies in adult females with TS are rare, occurring in approximately 2.0 - 7.6% of cases [13-17].

As the majority of females with TS is unable to conceive a child naturally, most of them depend on alternative parenting options such as adoption, foster care or oocyte donation. However, like most women, females with TS prefer a genetically-related child above other forms of parenting, as adoption, foster care or oocyte donation have their own limitations [18, 19]. The inability to bear biological children is the most prevalent and painful challenge experienced by most females with TS, especially at the moment that their family and friends begin to procreate [20, 21]. In an interview study with 97 females with TS aged 7 – 59 years and 21 parents, uncertainty about their fertility started at a young age and was a major concern for both groups [21]. In females with other causes of POI, uncertainty about fertility and the inability to have biological offspring was associated with a reduction of quality of life and serious psychosocial disorders [22].

It is, therefore, unsurprising that physicians are increasingly being asked about fertility preservation (FP) options for females with TS [23], especially as research shows that oocytes can still be found in the ovaries of some girls with TS [24-38].

FP is the process of safeguarding the patient's own gametes so that these can be used to have biological children in the future. In single females, FP can be performed by either the vitrification of mature oocytes or by cryopreserving ovarian tissue containing primordial follicles. Vitrification of mature oocytes (oocyte cryopreservation, OC) is the most established FP approach but is limited to a small percentage of females with TS only, i.e. those with a spontaneous menstrual cycle during adolescence or adulthood [39]. Furthermore, females interested in OC have to be emotionally mature enough to undergo the procedure, which involves a period of at least 2 weeks with daily hormone injections, frequent ultrasonographic monitoring, and transvaginal oocyte retrieval [39]. In view of these limitations, OTC appears to be a more promising technique to preserve the fertility of females with TS, as it can be performed regardless of the patient's age or ovarian activity. This procedure may offer more females with TS the possibility to store a number of primordial follicles before their disappearance [26].

Since 2002, OTC procedures have been performed experimentally in more than 100 young females with TS [24-31][ClinicalTrials.gov: NCT01410045]. Unfortunately,

optimal discriminative markers for the presence or absence of follicles are lacking, but there is a general agreement that females with a mosaic karyotype are the most likely group to have ovarian follicles [23, 26, 31, 39].

Thus far, there are no published records of girls with TS who have returned for auto-transplantation of cryopreserved ovarian tissue. Hence, the efficacy of OTC in females with TS remains unknown. In other patient groups, the occurrence of pregnancy and live birth after autotransplantation of cryopreserved ovarian tissue is highly correlated with the number of functional primordial follicles found in the ovarian tissue [40]. However, even in these patient groups there is limited data regarding the efficacy of the procedure when OTC is performed at a very young age [41]. Autotransplantation of ovarian cortex fragments with a decreased follicular density combined with the risk of re-initiation of accelerated follicle apoptosis might be less effective. Possibly, *in vitro* activation (IVA) of residual dormant follicles [42-45] prior to autotransplantation might be helpful in females with TS to optimize their fertility chances. However, no cases of IVA in patients with TS have been reported thus far. The isolation and IVM of primordial follicles from cryopreserved ovarian tissue could become an effective alternative to autotransplantation in the future [46]. However, this method is still experimental and not yet available in the clinic. Hence, females with TS who are currently undergoing OTC are still depending on autotransplantation.

Even if the follicular density is normal or slightly decreased, it remains questionable if autotransplantation of cryopreserved ovarian tissue in females with TS will lead to healthy offspring, as females with TS females who conceived spontaneously are known to have an increased risk of miscarriages and chromosomal abnormalities among their offspring [15]. Whether these increased risks are related to the quality and functional integrity, or the chromosome profile, of the follicular cells remains unclear.

Hence, offering OTC in routine clinical care could give false hope which could lead psychological harm in the future. Young patients might not be able to fully understand the possible risks and benefits of the procedure [47-49], and thus, parents will be burdened with this decision [50].

Another concern that should be taken into account is that OTC requires laparoscopic surgery under general anaesthesia with a possible risk of complications [51]. Furthermore, removing half of the ovarian reserve in females with TS might impact their chances for spontaneous puberty, menstruation and pregnancy. The short-term and long-term effects of the surgical removal of one ovary in females with TS are currently unknown, but recent studies [52-55] have shown that the surgical removal of one ovary in females with a normal ovarian reserve does not affect the patient's menstrual cycle, or their chance for spontaneous pregnancies in the future [56]. However, the procedure could lead to early menopause, of up to 3 years earlier in comparison to a woman who still has both ovaries [57].

Lastly, one should consider that pregnancies in females with TS show more foetal and maternal complications compared to pregnancies in healthy females. Pregnant females with TS have an increased risk of intra-uterine growth restriction and preterm labour [58]. Thyroid dysfunction, diabetes, obesity, hypertension and pre-eclampsia occur in approximately 40% of pregnant women with TS [58]. In the past, women with TS were advised to avoid pregnancy due to the risk of mortality. Recent studies have shown that the risk for aorta dissection and maternal mortality associated with pregnancy have decreased from 2.0% to 0.5% due to increased awareness of cardiovascular complications, stringent preconception screening, and cardiovascular follow up during pregnancy [5, 59]. Pregnancies in females with TS should be strictly monitored by a multidisciplinary team including high-care obstetricians, and cardiologists and anaesthesiologists with expertise in maternal heart disease and/or disease of the aorta. If this care is unavailable, pregnancies in females with TS might be contraindicated because of an increased risk of complications.

For the above mentioned reasons, FP in females with TS remains a controversial topic for clinicians [60]. However, patient organizations are optimistic and demand equal access to FP options worldwide [26, 47]. To further explore the opinion of international professionals and patient representatives, we conducted a three-stage ethical Delphi study to systematically discuss the advantages and disadvantages of OTC in females with TS. The aim of this study was to reach group consensus and to form an internationally accepted standpoint as to whether OTC should be offered to females with TS, or not.

Materials and methods

The RAND/UCLA Delphi procedure [61-63] was used to combine scientific evidence with the expertise and opinion of different international experts within the field of TS, OTC, or medical ethics. The Delphi procedure is a well-accepted method for attaining group consensus. It is a structured process that uses a series of questionnaires or rounds to gather information from different experts anonymously. Rounds are held until group consensus is reached according to predetermined defined consensus rules. In medical research, the Delphi procedure is commonly used to reach consensus on key recommendations [64], quality indicators [65], or key statements [66].

In this study, the Delphi procedure was used to determine whether the expected benefits exceed the expected negative consequences of OTC in TS [63, 67]. The outcome of this study was an international standpoint for or against OTC in females with TS, supported by a set of key statements.

Two questionnaire rounds and one agreement round were performed. Panel members were polled individually and anonymously. Participants were offered the possibility to

adjust their opinions after repetitive feedback after each round, thus avoiding the negative social influences associated with face-to-face discussion [62]. Questionnaires were conducted by electronic data capture, using *CastorEDC®* (Castor, George Westinghousestraat 2, 1097 BA, Amsterdam, The Netherlands). Possibilities to add new statements or comments were provided in each questionnaire. Invitations and reminders were sent via *CastorEDC®*. All scores were listed in a database created with IBM SPSS Statistics version 25.0 (IBM Netherlands, Johan Huizingalaan 765, P.O. Box 9999, 1066 VH Amsterdam, Netherlands). The consensus procedure took place between February and December 2018.

Identifying ethical issues and formulating statements

A total number of 65 articles were screened for arguments both for and against OTC in TS after a comprehensive literature search (**Figure 1**). Each article was screened independently by two researchers from a multidisciplinary research group (i.e. two gynaecologists (n=KF, CB), one paediatric endocrinologist (n=AvdV), one medical ethicist (EvL), one senior scientist in reproductive biology (n=RP) and one physician in reproductive medicine (MS) for arguments regarding OTC in females with TS. None of these researchers participated in the Delphi selection procedure. Arguments were extracted if both independent researchers agreed.

This selection procedure resulted in a total number of 155 arguments regarding OTC in TS. Arguments focusing on a similar topic were grouped and brought together into a framework of 38 statements (**Supplementary Table SI**). These statements were divided over the four basic ethical domains [68], i.e. beneficence (n=18), autonomy (n=5), non-maleficence (n=8), and justice (n=7). Each statement highlighted a specific ethical concern regarding OTC in females with TS. The original 155 arguments are presented as additional information below each statement.

Selection of key statements and formulation of a common stand

Step 1: Composition of the expert panel

To enhance the acceptance of this international standpoint in clinical practice, the expert panel consisted of a representative diversity of international professionals and patient representatives. Sufficient English language proficiency was an admission requirement for all experts. Invitations for the Delphi study were sent out by E-Mail to 59 international professionals (female professionals n=37, male professionals n=22) with expertise in the field of FP, TS, and/or medical ethics. Eligible experts were gynaecologists (n=18) and (paediatric) endocrinologists (n=17) with either a prominent role in one or more international expert groups (i.e. ESHRE Special Interest Group for Fertility Preservation, DSD-Life, Oncofertility, FertiPROTEKT, or Turner Syndrome Guideline Group), and/or an author of one or more key publications regarding FP in females with TS. Medical ethicists (n=24) were recruited by the ESHRE Task Force Ethics & Law and

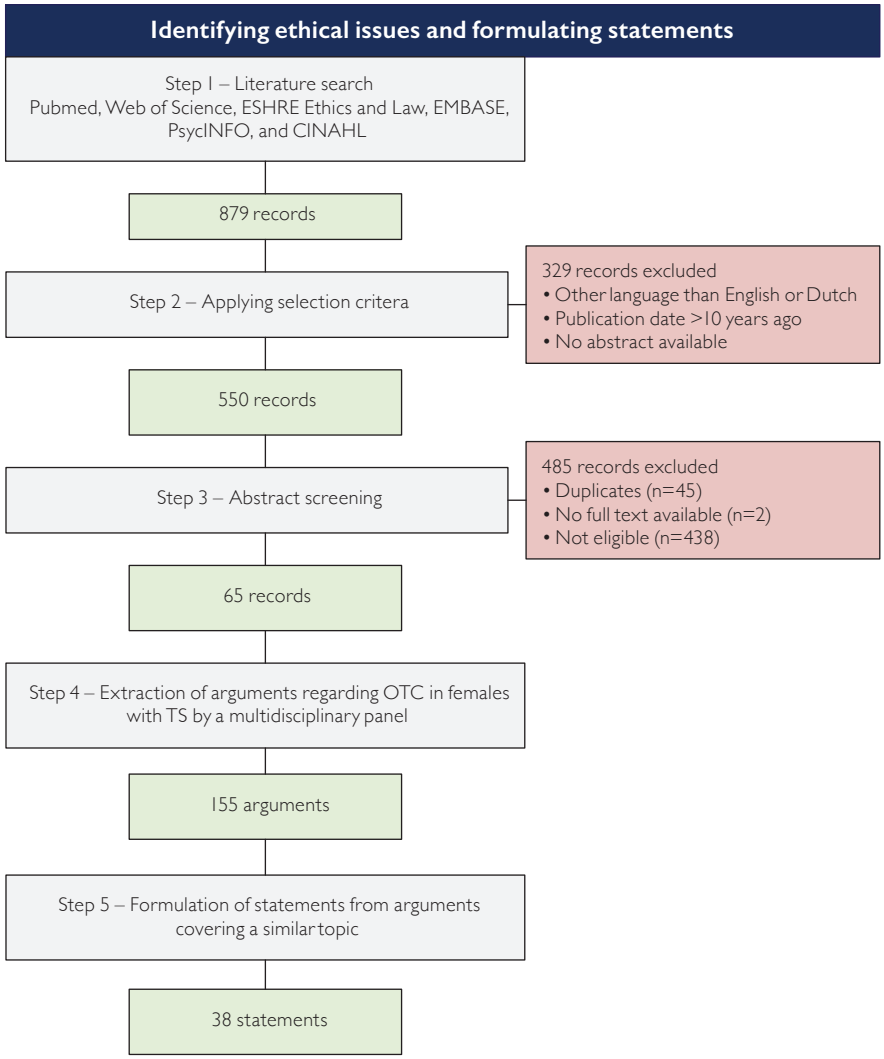


Figure 1. Identifying ethical issues and formulating statements. A detailed process description of the comprehensive literature search, extraction of arguments and formulation of statements.

the personal network of the senior research team members (KF, AvdV, EvL, WN, CB, RP, DB). Patient representatives should have had a prominent role in one of the international TS patient organizations. Therefore, a request for patient representatives (i.e. patients and/or parents of patients) was sent out to 17 patient organizations in 15

different countries. Contact data from 38 patient representatives were obtained, and invitations were sent out to them by E-Mail.

Step 2: First Delphi round

In the first Delphi round, the panel was asked to rate the 38 statements on a 9-point Likert scale ranging from 1 (extremely irrelevant) to 9 (extremely relevant). Relevance was graded by the experts in response to the following question; *"To what extent is the following statement an important determinant to offer/discourage OTC in young females with TS?"*. Participants were encouraged to read the original arguments and were provided with supporting evidence and background information. By the end of the questionnaire, participants were asked to create a top 5 of their most relevant statements to promote discrimination between recommendations with a high Likert score. In addition, they were given the option to add comments and new statements. Participants were given 4 weeks to complete the first round. Reminders were sent after 2 and 3 weeks.

The results of the first round were analysed using predefined consensus criteria based on Campbell's criteria [69]. These criteria include a median score of 8 or higher without panel disagreement. Panel disagreement was defined as the case in which 25% or more of the individual scores was in the lowest tertile of the scale (Likert score 1-3). Previous studies [70-77] have shown that Campbell's criteria alone are often not discriminative enough. Therefore, a third criterion was added, which is commonly used in Delphi studies, namely a top 5 score [70-73, 75-77]. Recommendations should have at least a top 5 score of 35 points or higher. Points were awarded to each top-five ranking position, with number 1 position = 5 points, number 2 position = 4 points, number 3 position = 3 points, number 4 position = 2 points, and number 5 position = 1 point. The authors combined the three criteria as described above and converted them into three possible outcomes 'selected', 'rejected', or 'no consensus'. Recommendations that met all three criteria were classified as 'selected', those who met none of the criteria as 'rejected', and the remaining recommendations as 'no consensus'. The 'no consensus' recommendations were again discussed in the second questionnaire round.

Step 3: Second Delphi round

The second round started with an overview of the selected, rejected and 'no consensus' recommendations. First, the experts were asked for their approval of the key statements that have been selected in the first round. Second, the expert panel was asked to revise their opinion for the 'no consensus' recommendations in light of the replies of the other panel members. The overall median score, the median score of each subgroup, the total top 5 score, and their own previous rating was shown for each statement. When experts were not able to participate in the first Delphi round (n=9), but wanted to participate in the second Delphi round, the overall median score, the median score of each subgroup, and the total top 5 score was shown for each statement. Participants

were asked once again to score the 'no consensus' recommendations on the same 9-point Likert scale as used in the first Delphi round (ranging from 1 (extremely irrelevant) to 9 (extremely relevant)). By the end of the questionnaire, the recommendations that they scored with 8 or higher were shown, and participants were asked to select up to three additional statements.
Participants were given 3 weeks to complete the second round. Reminders were sent after 1 week and after 2 weeks.
The selection of additional key statements during the second round was based on two predefined criteria that were used during the first round (i.e. a median score of 8 or higher and panel disagreement below 25%). Furthermore, additional key statements should be selected by at least 30% of the experts. These three criteria were combined and converted into two possible outcomes: 'selected' or 'rejected'. Recommendations that met all criteria were classified as 'selected' and the remaining recommendations as 'rejected'.
After having discussed the medical and ethical aspects of OTC in females with TS, the expert panel was asked to form a current standpoint if OTC should be offered to young females with TS or not.

Step 4: Final approval

An overview of the selected key statements and the current expert panel's standpoint was sent out by E-mail to all 55 experts who initially gave consent for study participation, as 14 out of the 55 experts (25%) did not participate in the second Delphi round. They were provided with a last opportunity to make remarks and asked for their approval of the final set of key statements and the expert panel's standpoint in order to reach international consensus.

Results

Selection of key statements and formulation of a common stand

Step 1: Composition of the expert panel

A total number of 12 gynaecologists, 13 (paediatric) endocrinologists, 10 medical ethicists and 20 patient representatives (patients n = 7, parents n = 13) from 16 different countries gave consent to participate in this study, forming an international expert panel of 55 members (**Figure 2**). The composition of the expert panel for each Delphi round has been visualized in **Supplementary Figure S2**.
All professionals, except one of the (paediatric) endocrinologists, were employed in academic hospitals or were working in an academic setting. Most of them were females (n=23), and about one-third of them were male professionals (n=12), Gynaecologists

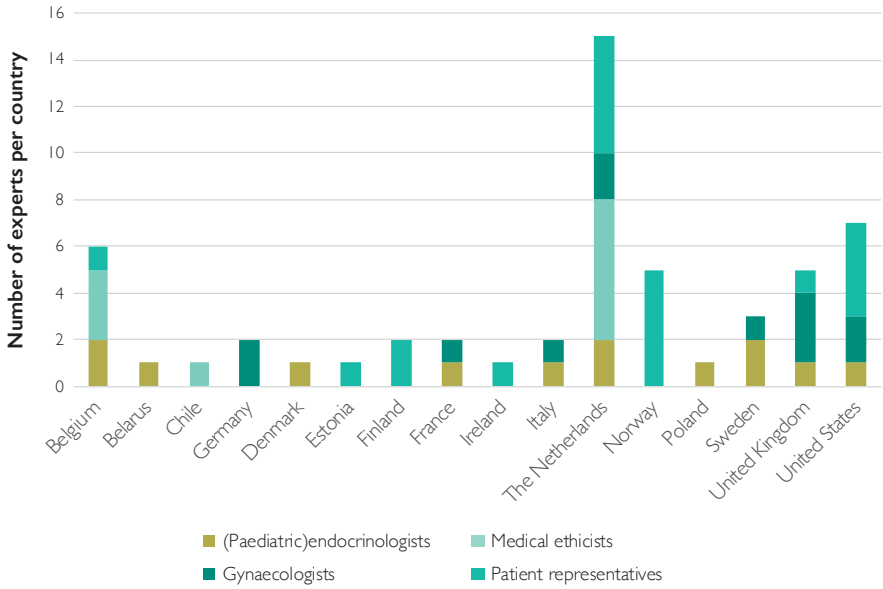


Figure 2. Members of the TurnerFertility expert panel (n=55) divided by country.
(Paediatric) endocrinologists (n=13) representing 10 countries: Belgium (n=2), Belarus (n=1), Denmark (n=1), France (n=1), Italy (n=1), The Netherlands (n=2), Poland (n=1), Sweden (n=2), United Kingdom (n=1), United States (n=1).
Medical ethicists (n=10) representing 3 countries: Belgium (n=3), The Netherlands (n=6), Chile (n=1).
Gynaecologists (n=12) representing 7 countries: Germany (n=2), France (n=1), Italy (n=1), The Netherlands (n=2), Sweden (n=1), United Kingdom (n=3), United States (n=2).
Patient representatives (n=20) (7 patients with TS and 13 parents) representing 8 countries: Belgium (n=1), Estonia (n=1), Finland (n=2), Ireland (n=1), The Netherlands (n=5), Norway (n=5), United Kingdom (n=1), United States (n=4).

had an average work experience of 23 years (range 9-40 years), (paediatric) endocrinologists had an average work experience of 21.4 years (range 7-40 years), and medical ethicists had an average work experience of 22 years (range 9-38 years).
Many professionals (77%) had children themselves, and most of them conceived spontaneously. Two professionals conceived using ART and one professional adopted a child.
The remaining panel consisted of 20 patient representatives, seven females with TS and 13 parents of patients with TS. The average age for patients was 32 years (range 18-50) and for parents 45 years (range 36-55).
Most patient representatives were highly educated, with at least an associate degree (n=15), while the remaining five patient representatives finished at least high school.

None of the women with TS had children themselves, but all except one expressed a desire to have children in the near future. None of them had tried ART. One woman with TS tried to adopt a child but was rejected for unknown reasons. One of the parents adopted a child, and the others conceived spontaneously.

Step 2: First Delphi round

A total number of 46 participants completed the first Delphi-round (response rate 84%). Reasons for not participating were: concerns about privacy (n=1), not enough time (n=4), not feeling sufficiently involved with the subject (n=2), a conflict of interest (n=1), and software problems (n=1). The average time for completing the first survey was 30 minutes.

Based on the predefined selection criteria, six of the 38 statements were selected as key statements (Fig. 3). These six key statements were divided over the four ethical domains (beneficence (n=2), autonomy (n=1), non-maleficence (n=2), and justice (n=1)). In the first round, 13 statements could be discarded, and 19 statements remained undetermined. In addition, two new statements submitted by the expert panel, were added to the second Delphi round (**Supplementary Table SI**).

Step 3: Second Delphi round

The remaining 19 statements and the two additional statements submitted by participants were re-evaluated in the second round by 41 participants (response rate 75%). Time investment was reported as the main reason for not participating. The average time for completing the second survey was 10 minutes.

The analysis of the second survey resulted in the inclusion of two additional key statements (**Figure 3**). The other 19 statements could be discarded.

By the end of the second Delphi round, 30 experts (75%) believed that OTC should be offered to young females with TS in a safe and controlled research setting, one participant voted against (2%), and 10 experts chose to remain neutral (24%).

Step 4: Final approval

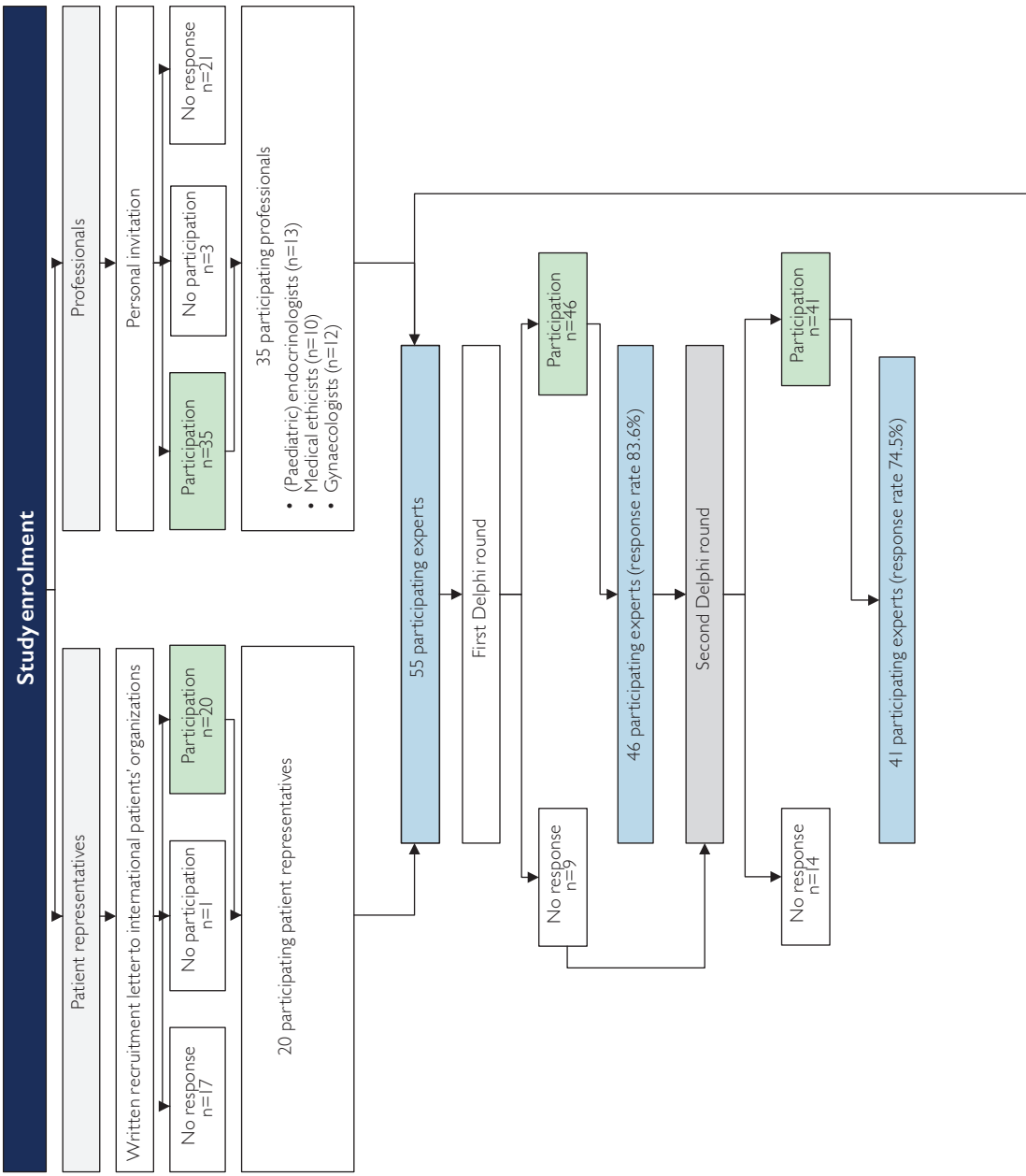
The final set of eight key statements (**Figure 3**) and the current expert panel's standpoint was approved by 53 out of 55 participants (response rate 96%). All experts, including those who voted against, remained neutral or did not respond in the second Delphi round, now agreed that OTC should be offered to young females with TS, but under strict conditions only. The expert panel suggested a research setting, with proper counselling and informed consent procedures, and a long-term follow-up to study the efficacy of OTC in young females with TS first, before offering this procedure in routine care.

Two experts (4%), one gynaecologist and one medical ethicist, did not respond despite several reminders.

KEY STATEMENTS	Delphi round	Median total	Median (paediatric) endocrinologists	Median medical ethicists	Median gynaecologists	Median patient representatives	Panel disagreement	Top 5 score	Selection percentage
Theme 1: Beneficence									
1. Infertility in women with Turner syndrome leads to psychological harm.	1	8	8	8	8	7.5	2.2%	46	-
2. Patients with Turner syndrome rate their own infertility as their primary concern.	1	8	8	8	7	8	4.3%	65	-
3. There are other ways to become a parent besides having genetically own children.	2	8	7.5	7	8	7.5	4.9%	-	30.5%
Theme 2: Autonomy									
4. Regardless their age, girls with Turner syndrome should be included in the consent process.	1	8	8	8	8	7.5	4.3%	38	-
Theme 3: Non-maleficence									
5. Pregnancies in women with Turner syndrome are associated with higher rates of maternal complications.	1	8	8	7	9	8	6.5%	52	-
6. Pregnant women with Turner syndrome are at risk of severe cardiac complications and mortality.	1	8	8	6	8	8	6.5%	60	-
Theme 4: Justice									
7. Patients at risk of premature ovarian insufficiency should have equal access to counselling and fertility preservation options.	1	8	8	8	9	8	6.5%	70	-
Additional statements									
8. Each girl with TS interested in OTC, should be discussed within a multidisciplinary expert team, after psychosocial and cardiologic screening have taken place.	2	8	8	7	9	7.5	7.3%	-	44.4%

Figure 3. The final set of 8 key statements divided over the 4 basic ethical themes (beneficence, autonomy, non-maleficence, and justice). Inclusion was based on predetermined selection criteria (i.e. a median of 8 or higher AND panel disagreement below 25%) AND a top 5 score of 35 points or higher (first Delphi round) OR a selection percentage of 30% or more (second Delphi round).

The detailed process of study enrolment and response rates by Delphi round is shown in **Figure 4**. A process description of the key statement selection by Delphi round can be found in **Supplementary Figure S2**.



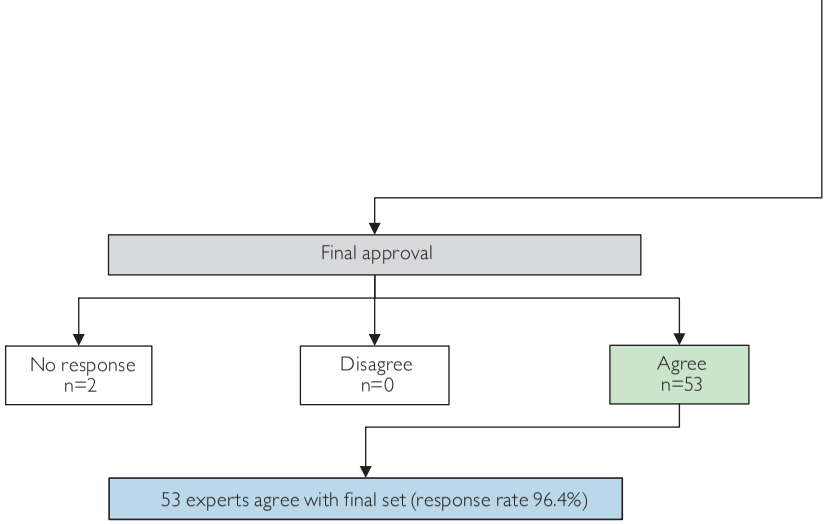


Figure 4. Detailed process of study enrolment and response rates per Delphi round.

Differences in scoring behaviour between experts

In the first Delphi round, patient representatives mainly highlighted arguments focusing on the psychological harm of infertility, and one of the key statements was selected by the patient representatives despite a moderate popularity among the professionals. This statement focused on the impact of infertility on the quality of life. Medical ethicists were more concerned about creating false hope resulting in psychological harm in the future. Gynaecologists and (paediatric-) endocrinologists underlined statements regarding non-maleficence. The fact that young TS patients might be too immature to give informed consent was considered a major issue for both patient representatives and (paediatric) endocrinologists but was not recognized by the gynaecologists and medical ethicists. Younger participants (<25 years old) expressed the importance of including all patients in the consent process, regardless of their age. The statement that 'infertility leads to psychological harm' was selected twice as frequently by participants without children compared to participants with children. Male participants were more concerned about pregnancy-related complications, whereas the female participants highlighted the psychological harm of infertility. Religion, age, and the availability of OTC in the participant's country did not influence the scoring behaviour between participants in the first Delphi round.

Differences in scoring behaviour between the various experts were only seen in the first Delphi round and disappeared in the following two rounds when opinions were exchanged.

Discussion

In this Delphi study, we demonstrated that an international expert panel of both professionals and patient representatives agreed that OTC in patients with TS should be offered, but in a safe and controlled research setting only. The expert panel's standpoint was supported by eight key statements (**Figure 3**). The first two statements highlighted that infertility leads to psychological harm, and that patients with TS consider this their main concern (key statements 1 and 2). In addition, patients with TS should have equal access to FP options, in line with other patient groups (e.g. patients awaiting cancer treatments) (key statement 7). However, there were concerns about the increased risk of maternal morbidity and mortality associated with TS during a future pregnancy (key statements 5 and 6). Patients with TS should be counselled about the alternative options for future parenthood (i.e. adoption, fostering, and oocyte donation) (key statement 3). Furthermore, the option of voluntary childlessness should be discussed. All patients with TS interested in OTC should undergo psychosocial and cardiac screening and should be discussed by a multidisciplinary expert team (key statement 8). Great caution and restrictive (i.e. more negative, or even discouraging)

counselling are recommended if laparoscopic surgery or pregnancy is contraindicated (i.e. in patients with severe cardiac comorbidity).
In addition, patients with TS should always be included in the consent process, regardless of their age (key statement 4).
To our knowledge, this is the first international standpoint regarding OTC in females with TS. To reach international group consensus we used the RAND/UCLA Delphi procedure, which is a well-accepted method to perform Delphi studies. A key strength of this study was the combination of evidence and expert opinion, involving both professionals, and patient representatives from 16 different countries. Furthermore, our expert panel represented a robust sample of the most important stakeholders to ensure that all the medical and ethical aspects of OTC in females with TS were discussed. The literature shows that a diversity of expert panel members leads to the inclusion of different perspectives, in turn leading to better overall performance [78]. This diversity provided a suitable set of key statements and consensus on a final standpoint, which should support broad acceptance in daily practice internationally. Remarkably, ours was one of the few studies where a combined panel of medical professionals and patient representatives was involved in defining a standpoint regarding the indication for medical treatment for a specific patient group. It is well known that patient input is invaluable when it comes to clinical practice guideline development [74, 75, 79]. Essentially, patients are the ultimate experts in patient-centeredness of care [80, 81], which is possibly the dominant paradigm in modern health care systems. The final set of key statements, and thus the outcome of this Delphi study, could have been different if only professionals had been involved in the selection procedure [73, 74, 82-85]. In the first Delphi round, patient representatives mainly highlighted arguments focusing on the psychological harm of infertility, whereas medical ethicists were more concerned about creating false hope resulting in psychological harm in the future. Gynaecologists and (paediatric) endocrinologists underlined statements regarding non-maleficence. This is in line with previous studies reporting that professionals underestimate 'softer' dimensions of healthcare (e.g. quality of life) and overestimate the importance of biomedical outcomes compared to patients [85-89].
However, differences in scoring behaviour between the various experts were only seen in the first Delphi round and disappeared in the following two rounds when opinions were exchanged. Only one of the key statements (*Infertility in women with TS leads to psychological harm)* was selected by the patient representatives, despite having a moderate popularity among the professionals.
Although we considered the expert panel to be representative because of their diverse backgrounds [82, 90-92], the recruitment of professionals and patient representatives based on their expertise, having a leading role in one of the Turner patient's organizations, and willingness to participate may have led to selection bias. As a result, panel members with a prominent opinion regarding OTC in females with TS might have been preferentially

motivated to participate in this Delphi study. Furthermore, as not all panel members took part in all three rounds, there might be some response bias [93] because of time constraints or technical problems. Therefore, the final set of key statements and the expert panel's standpoint might reflect the opinion of the most motivated panel members [93]. However, we tried to overcome this by inviting all 55 experts who initially agreed to participate in this Delphi study to participate in the final approval round.

The anonymous nature of this study was both a strength and a weakness. The purpose of anonymity in a Delphi study is to allow a safe exchange of opinions, without the bias of the more influential responders dominating the discussion. External influences are eliminated, as participants do not have to worry about their reputation. However, it might encourage hasty decision-making and a lack of accountability for their answers.

This study described the systematic selection of key statements and the formulation of a final standpoint regarding use of OTC in females with TS by an international panel of patient representatives and professionals. International group consensus was reached after three rounds. The approval to perform OTC in a safe and controlled research setting is the first step in the global acceptance of this FP option in females with TS. The eight supporting key statements will contribute to the implementation of and patients' access to this new treatment method. Our results reinforce the importance of involving patient representatives in decision making and guideline development.

Future collaborative research with a focus on the efficacy and safety of OTC in females with TS is urgently needed before OTC is performed in females with TS in routine care. Therefore, we recommend an international prospective cohort study with long-term follow-up with a research protocol based on the eight selected key statements. Furthermore, we recommend an international register for FP procedures in females with TS.

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Supplementary Table S1. Overview of the 38 statements divided over the four basic ethical themes that formed the basis of this Delphi study. In the first Delphi round 2 new statements were added (statement 39 and 40).

THEME 1: BENEFICENCE

1. Infertility in women with Turner syndrome leads to psychological harm.
2. Patients with Turner syndrome rate their own infertility as their primary concern.
2. The risk of infertility in patients with Turner syndrome has a negative impact on their intimate relationships and their chances at marriage.
4. Fertility is an essential element of femininity and is linked to a woman's sense of identity.
5. There are other ways to become a parent besides having genetically own children.
6. Most women prefer biologic offspring.
7. It is more difficult for women with Turner syndrome to adopt a child.
8. Live birth rates are doubled in woman with Turner syndrome who used donor oocytes to become pregnant.
9. Oocyte donation in women with Turner syndrome leads to significantly less chromosomal abnormalities in their offspring
10. Intrafamilial oocyte donation offers woman with Turner syndrome an option for genetically related offspring with reduced foetal risks.
11. Intrafamilial oocyte donation could lead to imbalances in the relationship between patient, donor and child.
12. Cryopreservation of oocytes is an established fertility preservation method for woman and post-pubertal girls, whereas ovarian tissue cryopreservation is still experimental.
13. Cryopreservation of oocytes requires ovarian stimulation with exogenous FSH, whereas ovarian tissue cryopreservation does not.
14. Cryopreservation of oocytes requires transvaginal ultrasound guided oocyte retrieval, whereas ovarian tissue cryopreservation does not.
15. Cryopreservation of oocytes can be performed only in girls who have had a spontaneous menarche, whereas ovarian tissue cryopreservation can be performed in both pre- and post-pubertal girls.
16. Ovarian tissue preservation has been demonstrated as a successful fertility preservation option in other patient groups.
17. The germ cell loss will continue after auto transplantation of cryopreserved ovarian tissue.
18. The attempt to preserve a patient's fertility decreases the patient's psychological distress and promotes her psychological comfort.

THEME 2: AUTONOMY

19. Most patients diagnosed with premature ovarian insufficiency express a desire for future offspring.
20. Patients and parents are motivated to try experimental fertility preservation methods.
21. Young patients with Turner syndrome might be too immature to give informed consent for experimental fertility preservation methods.

Supplementary Table S1. Continued.

THEME 2: AUTONOMY

19. Most patients diagnosed with premature ovarian insufficiency express a desire for future offspring.
20. Patients and parents are motivated to try experimental fertility preservation methods.
21. Young patients with Turner syndrome might be too immature to give informed consent for experimental fertility preservation methods.
22. Regardless their age, girls with Turner syndrome should be included in the consent process for ovarian tissue cryopreservation.
23. Fertility preservation could create expectations for the child to reproduce.

THEME 3: NON-MALEFICENCE

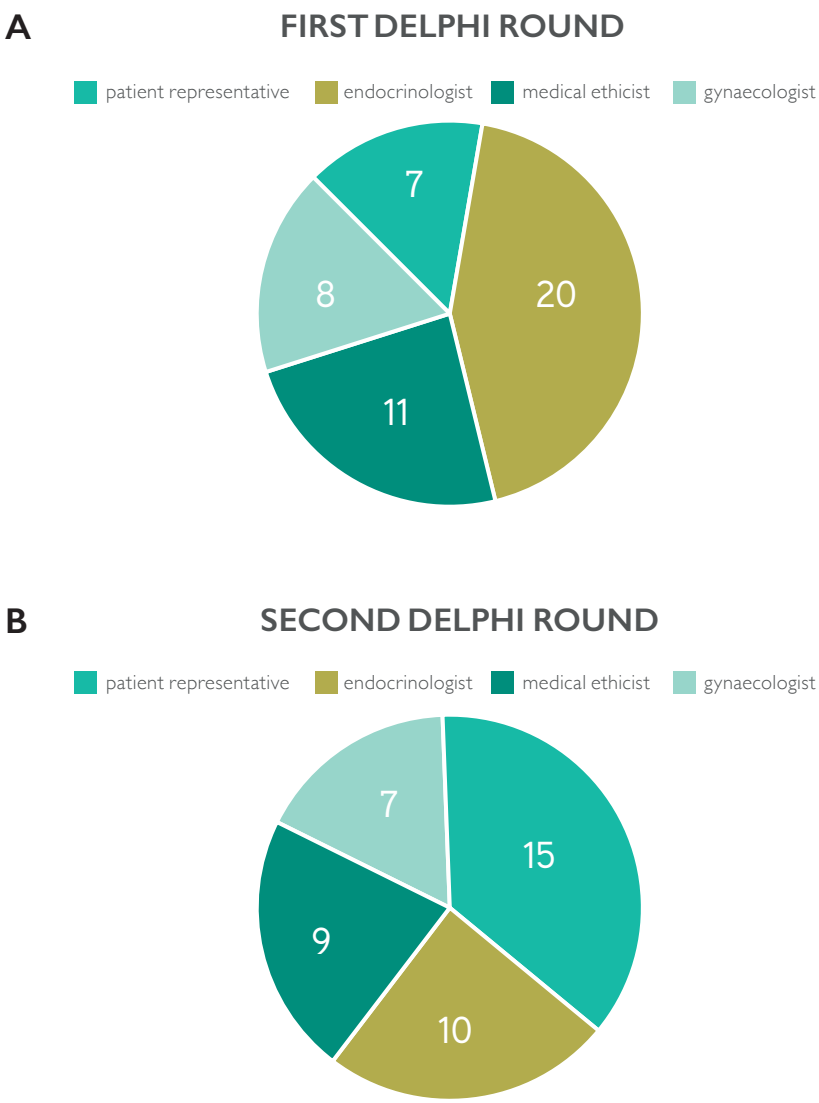
24. Pregnancies in women with Turner syndrome show higher rates of foetal complications.
25. Oocyte genetics or pre-implantation genetic screening can be used to screen oocytes and embryos.
26. Pregnancies in women with Turner syndrome show higher rates of maternal complications.
27. Furthermore, pregnant women with Turner syndrome are at risk of severe cardiac complications and mortality.
28. Ovarian tissue cryopreservation requires laparoscopic surgery.
29. Experimental fertility preservation options could create false hope, which could lead to psychological harm in the future.
30. A decisional conflict between the child and the parents could cause harm to their relationship.
31. Performing ovarian tissue cryopreservation in paediatric patients upon parents' consent, may lead to both positive and negative feelings during adolescence of adulthood.

THEME 4: JUSTICE

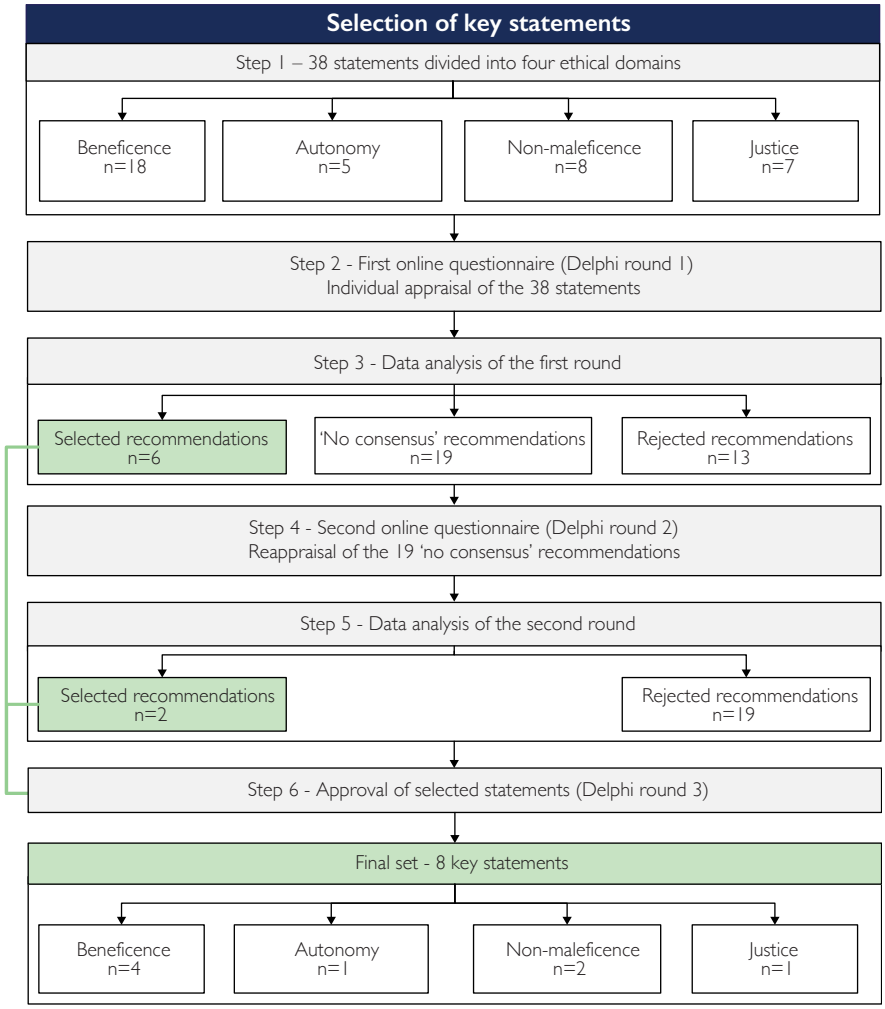
32. Reproduction is a fundamental human right.
33. Patients at risk of premature ovarian insufficiency should have equal access to counselling and fertility preservation options.
34. Fertility preservation is a typical instance of 'medicalization'.
35. Ovarian tissue cryopreservation is also performed for non-medical reasons and social indications.
36. In some countries, ovarian tissue cryopreservation is already experimentally offered to girls with Turner syndrome.
37. Ovarian tissue cryopreservation remains financially out of reach for some patients.
38. Girls with Turner syndrome are less likely to get sexual education from their parents, due to their infertility.

ADDITIONAL STATEMENTS

39. Each girl with TS interested in OTC, should be discussed within a multidisciplinary expert team, after psychosocial and cardiologic screening have taken place.
40. Fertility preservation options might make us unhappier in the long run, because they contribute to a society where tragedy and human limitations are not accepted and are insurmountable problems.



Supplementary Figure S2. Participants of per Delphi round divided by subgroup. A. Participants of the first Delphi round (n=46) divided by subgroup; (paediatric)endocrinologists (n=11), medical ethicists (n=8), gynaecologists (n=7), and patient representatives (n=20). B. Participants of the second Delphi round (n=41) divided by subgroup; (paediatric)endocrinologists (n=10), medical ethicists (n=9), gynaecologists (n=7), and patient representatives (n=15).



Supplementary Figure S3. Detailed process description of the selection of key statements in this 3-round Delphi study.

5

Clinical aspect

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TurnerFertility Trial: PROTOCOL for an Observational Cohort Study to Describe the Efficacy of Ovarian Tissue Cryopreservation for Fertility Preservation in Females with Turner Syndrome.

BMJ Open. 2019;9(12):e030855

Abstract

Objective

To investigate the occurrence of live birth in women with Turner Syndrome (TS) after ovarian tissue cryopreservation in childhood followed by auto transplantation in adulthood and to find reliable prognostic markers for estimating the ovarian reserve in girls with TS in the future.

Setting

An observational cohort study with long-term follow-up in a tertiary fertility clinic in the Netherlands. Patients recruitment between January 2018 and December 2021.

Participants

100 females aged 2 through 18 years with classical Turner (i.e. 45, X) or Turner variants (i.e. 45, X mosaicism or structural anomalies). Girls with Y chromosomal content, minor X deletions with marginal impact on fertility, active HIV, hepatitis-B or hepatitis-C infection, and/or an absolute contra indication for surgery, anaesthesia or future pregnancy will be excluded.

Interventions

Ovarian cortical tissue will be harvested by performing a unilateral oophorectomy via laparoscopic approach. Ovarian cortex fragments will be prepared and cryopreserved. One fragment per patient will be used to determine follicular density by conventional histology, and to perform FISH analysis of ovarian cells. Routine chromosome analysis will be performed on both lymphocytes and buccal cells. A blood sample will be taken for hormonal analysis and all subjects will undergo a transabdominal ultrasound to determine the uterine and ovarian size. Patient characteristics, pregnancy rates, and pregnancy outcomes will be collected from the patient's medical record.

Ethics and dissemination

The study protocol has been approved by the Central Committee on Research Involving Human Subjects in November 2017 (CCMO NL57738.000.16).

Registration details

This study is registered in ClinicalTrials.gov (NCT03381300).

Strengths and limitations

- Patients were involved in the initiation and design of this study protocol.
- The long-term follow-up of this study provides the unique opportunity to study the efficacy of ovarian tissue cryopreservation and pregnancy outcomes in females with TS.
- The single-centre design could be a limitation.

Background

Turner Syndrome (TS) is the most common chromosomal abnormality among females, affecting 1 in 2,500 live born girls [1-3]. Due to the partial or complete absence of one of the two X-chromosomes the foetal development is affected, leading to abnormalities in almost all organs including the gonads [4]. Signs and symptoms vary among girls with TS, and intelligence is generally normal. The most common phenotypic features are infertility, short stature and cardio-aortic malformations.

Females with TS are known to have a limited reproductive lifespan due to an accelerated loss of germ cells. This process starts during the fatal period and continues until the point when the ovarian reserve is empty [5, 6]. Previous research has shown that primordial follicles can still be found in the ovaries of young girls with TS [7]. However, in most females with TS the ovarian reserve is exhausted before reaching adulthood [6]. Approximately one-third of females with TS have some pubertal development and 10-15% will experience one or more spontaneous menstruation cycles [8-10]. Spontaneous pregnancies occur in approximately 2.0-7.6% of women with TS [11-15]. Several interview studies [16, 17] show that, regardless of age, uncertainty about their fertility is one of the major concerns for girls and females with TS and their parents. While fertility preservation has garnered greater attention in the media, physicians are frequently asked whether these methods could also be used to preserve the fertility of patients with TS.

Fertility preservation includes the cryopreservation of the patient's own gametes, either by preserving mature oocytes or ovarian tissue containing primordial follicles. Cryopreservation of mature oocytes (OC) is a proven fertility preservation approach but requires ovarian activity, a good ovarian reserve, and psychological maturity [18]. This method is limited to a small percentage of females with TS, namely those who will be fertile after a spontaneous onset of puberty and menstruation. Furthermore, the patient has to be emotionally mature enough to undergo the procedure, which involves ovarian stimulation with exogenous FSH administration followed by transvaginal ultrasound-guided oocyte retrieval [18].

Because of the limitations of OC in girls with TS, ovarian tissue cryopreservation (OTC), appears to be a more promising technique for fertility preservation for this condition. The procedure can be performed in patients with TS regardless of their age or ovarian activity, and probably offer more females with TS the possibility to store a number of primordial follicles before their disappearance [19]. OTC is a proven method to preserve the fertility of young females at risk of iatrogenic premature ovarian insufficiency (POI) such as females undergoing gonadotoxic cancer treatments [20-22]. Auto transplantation of cryopreserved-thawed ovarian cortical tissue in cancer survivors has resulted in restoration of ovarian function in 67-93% of cases [23-26] with reported live birth rates between 25-33% per transplantation [24, 25, 27, 28]. A recent

study with a limited number of patients even reported a live birth rate up to 75% [26]. Over the past decades, several clinical guidelines [29-38] and decision tools [39-44] for ovarian tissue cryopreservation have been developed for these patients.
From 2002, OTC procedures have also been performed experimentally in at least 83 young females with TS [7, 19, 45-47]. In approximately one quarter of females with TS, follicles were present [19]. Unfortunately, optimal discriminative markers for the presence or absence of follicles in females with TS are currently lacking. However, there is a general agreement that the mosaic karyotype is the most promising group to have ovarian follicles and to benefit from fertility preservation [18, 19, 48, 49].
Furthermore, there are to date no published records of girls with TS who have returned for autotransplantation. As long as the efficacy of fertility preservation in females with TS regarding future pregnancy and live birth is unknown, experts might be reluctant to recommend routine OTC procedures in females with TS [18, 19, 50, 51].
Exploring the efficacy of fertility preservation in females with TS seems a logical next step [48]. There is a strong need for a structured observational cohort study with long-term follow-up. This study should focus on the efficacy of OTC in females with TS including pregnancy rates and pregnancy outcomes, and on the development of a reliable prognostic model for estimating the ovarian reserve in females with TS. Ideally, a decision aid based on the information needs of females with TS and their parents should be developed with the aim to help them to make a deliberate decision between OTC and the alternative options for future parenthood. This decision aid should include a reliable prognostic model to estimate the ovarian reserve in females with TS. Herewith, the urgency of fertility preservation could be determined and unnecessary surgical procedures can be avoided.

Objective

To investigate the occurrence of live birth in women with TS after OTC in childhood followed by auto transplantation in adulthood and to find reliable prognostic markers for estimating the ovarian reserve in girls with TS in the future.

Methods

Study design

An observational cohort study with long-term follow-up in a tertiary fertility clinic in the Netherlands. This study is registered in ClinicalTrials.gov (NCT03381300). The study flow is visualised in **Figure 1**.

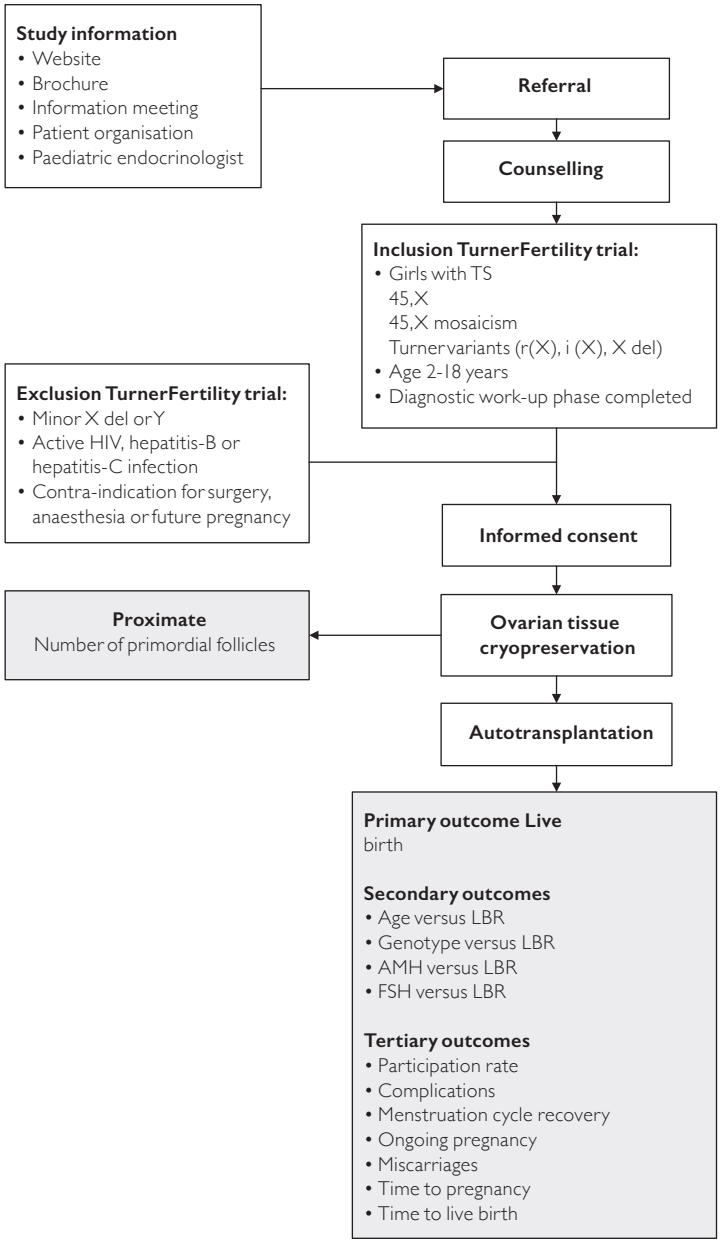

Figure 1. Flowchart of study. TS, Turner Syndrome; 45, X, 45, X Monosomy; r(X), ring chromosome X; if(X), isochromosome X; X del, X deletions; HIV, Human Immunodeficiency Virus; LBR, live birth rate; AMH, Anti Müllerian Hormone; FSH, Follicle Stimulating Hormone.

Study period

Recruitment started January 2018. OTC procedures will be performed between January 2018 and December 2021. We expect to end this study in 2071.

Interventions

Based on the international Cincinnati Turner Guideline Consensus Meeting, July 2016 [52] and the consultation of Dutch cardiologists, paediatric cardiologists and anaesthesiologists between 2016-2017, there are no absolute cardiovascular contra-indications for surgical intervention and/or pregnancy. Advice against surgical intervention and/or pregnancy should be based on the patient-specific cardiovascular risk profile.

Therefore, all girls will undergo preoperative screening and risk assessment by a paediatric cardiologist and paediatric anaesthetist. After this screening, they will be defined as either low-risk participants or high-risk participants based on their individual risk profile. High-risk patients and their parents will be informed about their risks and excluded from this study in order to ensure patient safety. In low-risk participants the surgical procedure, followed by a hospital stay of one night, will be planned.

Ovarian cortical tissue will be harvested by performing a unilateral oophorectomy via laparoscopic approach. A collaborating team of well-trained paediatric surgeons, gynaecologists specialised in reproductive medicine, and laboratory workers specialised in human ovarian cortical tissue cryopreservation, will ensure safe and efficient cryopreservation of the ovarian tissue. Cryopreservation of the ovarian tissue fragments will be performed according to the Dutch protocol 'Cryopreservation and transplantation of ovarian tissue' (Dutch Network Fertility preservation, September 2012).

One cortex fragment per patient of approximately 8x5x1mm will be used to determine follicular density by conventional histology, and to perform fluorescence *in situ* hybridisation (FISH) analysis of 3 different ovarian cell types (oocytes, granulosa cells and stromal cells) [53]. Routine chromosome analysis will be performed on both lymphocytes and buccal cells. If follicles are present, additional FISH analysis of urine cells will be performed. Furthermore, a blood sample of 3.5 mL will be taken for hormonal analysis (i.e. FSH, LH, AMH, oestradiol and inhibin B). All subjects will undergo a transabdominal ultrasound to determine the uterine and ovarian size. Information such as the patient's age and the spontaneous onset of puberty and menstruation will be collected from the patient's medical record. In the future, orthotropic transplantation of the auto graft is performed via laparoscopic or laparotomic approach [21, 54-56]. Pregnancy rates and outcomes will be collected from the patient's medical record.

Study population

The study population includes 100 females with TS.

Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

- Girls and young females with classic Turner (i.e. 45, X monosomy) or Turner variants (e.g. 45, X mosaicism, ring X, isochromosome X, X deletions),
- Aged 2 through 18 years,
- Who completed the diagnostic work up phase of TS, according to the international guidelines, including routine cardiac screening,
- Whose agreement to participate in this study has been signed by both the parents (girls 2-11 years old),
- Whose agreement to participate in this study has been signed by the patient and her parents (girls 12-15 years old),
- Whose agreement to participate in this study has been signed by the patient (girls 16-18 years old).

Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- Girls and young females with minor X deletions with marginal impact on fertility or Y chromosomal content.
- Girls with an active HIV, hepatitis-B or hepatitis-C infection.
- Girls with an absolute contra-indication for undergoing a laparoscopic unilateral oophorectomy under general anaesthesia and/or future pregnancy based on the patient specific risk profile (e.g. severe cardiovascular co morbidity and/or BMI >40 kg/m^2).

Recruitment of participants and informed consent

Girls with TS and their parents will be informed about this study by their paediatrician or by the Dutch TS patient organisation (Turner Contact Nederland). Girls with TS who meet the inclusion criteria and are potentially interested, will receive the study information for patients and/or parents (https://www.radboudumc.nl/trials/turner) and will be invited for a general informative meeting. If the girls / parents are still interested after receiving this information, they will be referred to our hospital by their paediatric endocrinologist by using a specific form designed for this study. The personal counselling is done by a dedicated team of gynaecologists. A paediatric psychologist will be available for additional support. In case of comorbidity, a multidisciplinary team of gynaecologists, (paediatric)cardiologists and other specialists if needed, will discuss the patient's specific risk profile.

Written consent must be given by the (parents of) patients before study participation. Girls and their parents are given time as individually requested to consider participation in this study. A pilot decision aid, website and age-specific information flyers have been

developed, to help girls and their parents to make a deliberate decision (https://www.radboudumc.nl/trials/turner). The current decision aid includes a flowchart and background information on OTC in comparison with the existing options (i.e. awaiting spontaneous pregnancy, vitrification of oocytes, oocyte donation, adoption and/or foster ship). When a participant reaches the age of 16 years old, she will be asked for reconsent.

Bio banking

All biological material and data will be handled and stored according to the World Medical Association (WMA) Declaration of Taipei on ethical considerations regarding health databases and biobanks (67th WMA General Assembly. Taipei, Taiwan. October 2016). All patient data will be coded. Members of the research team, the Data and Safety Monitoring Board, and the Health Care Inspectorate are the only persons who have access to the key of the code. This key of code is stored in Castor Electronic Data capture (EDC). The dignity, autonomy and privacy of the patients will be respected by the duty of confidentiality of all who are involved in handling data and biological material. There will be no discrimination.

Castor Electronic Data Capture (EDC) will be used for Good Clinical Practice (GCP)-compliant data collection. All participant data will be reported in electronic case report forms (e-CRF).

A safe storage of the ovarian tissue and consent forms is provided at our cryobank. This cryobank is ISO-accredited (accreditation number M101, ISO 15189), and registered by the Dutch Ministry of Health, Welfare and Sport (VWS) (registration number 5515 L/EO). The cryobank is located at a restricted area in the Radboud University Medical Centre, and access is permitted by electronic authorization only.

All subjects will be asked for the continuation of the storage of the ovarian tissue yearly. Each individual patient and/or her parents may freely decide about the continuation of the storage of the ovarian tissue at our cryobank, transportation of the ovarian tissue to another cryobank, donation for research, or the elimination of their cryopreserved ovarian tissue. When the participant turns 16 years old, she will be asked for re-consent. Subjects may withdraw from the trial at any time. Subjects do not need to state a reason for withdrawal.

Patient and Public Involvement

This study protocol has been initiated by and conducted with active input and feedback of international experts and patient representatives from the Dutch national patient organization Turner Contact Nederland (TCN) and the Differences of Sex Differentiation (DSD) patient advisory group of the Radboud university medical centre. Patient representatives were also involved in the development of patient information brochures, informed consent forms, and website. During the trial, patient representatives will be

involved by the development of a decision aid and newsletters. Furthermore, surveys and focus groups among all counselled patients are held for continuous process improvement. All participants will be informed of the results of the TurnerFertility study through post or email.
International consensus to perform this study was achieved in a Delphi study including 35 medical professionals and 20 patient representatives from 16 different countries. This expert panel stated that ovarian tissue cryopreservation (OTC) in patients with Turner syndrome (TS) should be offered, but in a safe and controlled research setting only. The expert panel's standpoint was supported by eight key statements [Unpublished data].

Outcome measures

Primary outcome

- Live birth after auto transplantation of cryopreserved-thawed ovarian cortical tissue (i.e. live birth rate or LBR)

Proximate

- The number of primordial follicles found in the ovarian tissue

Secondary outcomes

- The association between patient's age at cryopreservation and LBR
- The association between patient's genotype and LBR
- The association between patient's AMH level at cryopreservation and LBR
- The association between patient's FSH level at cryopreservation and LBR

Tertiary outcomes

- The study participation rate
- The number of eligible participants
- The age of the participant
- The incidence of somatic mosaicism
- The incidence of germ cell mosaicism
- Serum hormone levels
- The number of complications related to the laparoscopic procedure
- The incidence of spontaneous puberty and/or spontaneous menarche after laparoscopic oophorectomy
- The incidence of spontaneous pregnancies after laparoscopic oophorectomy
- The incidence of menstruation cycle recovery after auto transplantation of cryopreserved-thawed ovarian tissue in the future

- The incidence of pregnancies after auto transplantation of cryopreserved-thawed ovarian tissue in the future
- The number of ongoing pregnancies after auto transplantation of cryopreserved-thawed ovarian tissue in the future
- The number of miscarriages after auto transplantation of cryopreserved-thawed ovarian tissue in the future
- The incidence of congenital anomalies in the offspring of women with TS who became pregnant after auto transplantation of cryopreserved-thawed ovarian tissue
- Time to pregnancy after auto transplantation of cryopreserved-thawed ovarian tissue in the future
- Time to live birth after auto transplantation of cryopreserved-thawed ovarian tissue in the future

Data analysis

All data in this pilot study will be analysed on both intention-to-treat and per-protocol analysis. Data of girls who are lost to follow-up will be included as far as possible. Missing data will be reported along with the reason. Baseline data will be described quantitatively. Continuous variables will be summarised as means with standard deviations (SDs) or as medians with inter-quartile ranges (IQRs), depending on their distribution. Dichotomous and ordinal data will be summarised as percentages. For all analyses, IBM SPSS will be used.

Descriptive statistics will be used to analyse the number of primordial follicles in the ovarian tissue, and the number of (ongoing) pregnancies, miscarriages and live born children after auto transplantation of cryopreserved-thawed ovarian tissue.

The primary outcome (dichotomous) 'live birth after auto transplantation of cryopreserved-thawed ovarian tissue' will be assessed and reported as a percentage. The number of primordial follicles (continuous) will be reported as a mean with standard deviations (SDs). The number of pregnancies, ongoing pregnancies, miscarriages, and congenital anomalies (dichotomous) will be reported as a percentage.

The time to pregnancy and the time to live birth after auto transplantation will be reported as a mean with standard deviation (SD).

Furthermore, the relationship between the age of the participant when ovarian tissue cryopreservation (OTC) is performed (years), serum FSH level (IU/L), and serum AMH level (ng/ml), related to the number of primordial follicles, the incidence of (ongoing) pregnancies, live birth, and congenital anomalies will be described using Spearman's correlation coefficient. In addition, the number of primordial follicles, the incidence of (ongoing) pregnancies, live birth, and congenital anomalies will be described by the patient's karyotype (i.e. 45, X monosomy or mosaicism).

Interim analyses

Interim analyses on safety and futility are planned every 6 months until the last participant (n=100) has undergone the laparoscopic unilateral oophorectomy followed by ovarian tissue cryopreservation. The first interim analysis will be performed after the inclusion of the first 10 patients. Each interim analysis will be reported to the independent DSMB (*Data and Safety Monitoring Board*). The percentage of participants with follicles in their ovary will be used as a futility indicator. The percentage of participants who had one or more complications related to the laparoscopic oophorectomy and/or anaesthesia will be used as a safety indicator.

Sample size calculation

The estimated LBR related to OTC in girls with TS is currently unknown. Hence, the sample size calculation should be based on LBR in other patient groups. The LBR per transplantation of earlier cryopreserved ovarian tissue in cancer survivors is approximately 25% [21]. In order to describe the dichotomous outcome live birth rate (LBR), we used the sample size calculation of Hulley et al. 2013 [57]. We would need a total sample size of 72 participants if the LBR in females with TS would be similar.
However, one should consider that auto transplantation will not be performed in all girls who are participating in this study, due to several reasons (e.g. absence of follicles, future contra indications and/or on patient's preference). Furthermore, females with TS show an higher risk of miscarriage [13], and a slightly higher risk of a child with a congenital disorder when they conceive spontaneously [13, 58]. If these increased risks are related to the functional integrity or the chromosome profile of their follicular cells remains unclear. On the other hand, performing an exploratory intervention study in >100 minors, is unrealistic and inappropriate. Therefore, we aim to include primarily a total number of 100 girls with TS in this 'proof of concept' study.

Discussion

This study is an example of patient-initiated research as there is an increasing demand for fertility preservation options in girls and young females with TS [48]. This study protocol was developed together with patient representatives and gives females with TS the possibility to undergo an experimental fertility preservation procedure in a safe and controlled research setting. The long-term follow-up of this study up to live birth provides the unique opportunity to study the efficacy of ovarian tissue cryopreservation and pregnancy outcomes in females with TS. Furthermore, this study could contribute to the development of a reliable prognostic model for estimating the ovarian reserve in females with TS in the future.

A limitation of this study could be the single-centre design combined with the relatively low expected live birth rate. For the development of a prediction model, more patients should be included in the analysis to increase the internal validation. Furthermore, an external data validation should be performed. We suggest an international, multicentre study with a clear study protocol and a central registration after the results of the interim analysis of this pilot study have been published and are open for collaborating with other experts within the field of fertility preservation in females with TS.

Ethics and dissemination

This study protocol was conducted with input and feedback of patient representatives and international experts. Ethical approval by the Dutch Central Committee on Research Involving Human Subjects was obtained in 2017 (CCMO NL57738.000.16) and is in accordance with the Declaration of Helsinki, the Medical Research Involving Human Subjects Act (WMO), the Guideline for Good Clinical Practice, and all other applicable regulatory requirements. An independent Data and Safety Monitoring Board has been established to perform interim analyses on safety and futility. Results will be disseminated through peer-reviewed publications and presentations at international scientific meetings.

Ethics approval

Approved by the Dutch Central Committee on Research Involving Human Subjects (2017).

Data sharing statement

This study protocol including the ethical approval and informed consent forms will be available upon request. Individual participant data that underlie the study outcomes will be shared after deidentification. Requests should be addressed to the corresponding author.

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6

Genetic aspect

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Ovarian Follicles of Young Patients with Turner's Syndrome Contain Normal Oocytes but Monosomic 45, X Granulosa Cells.

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Abstract

Study question
What is the X chromosomal content of oocytes and granulosa cells of primordial / primary (small) follicles and stromal cells in ovaries of young patients with Turner's syndrome (TS)?

Summary answer
Small ovarian follicles were detected in one-half of the patients studied, and X chromosome analysis revealed that most oocytes were normal, granulosa cells were largely monosomic, while stromal cells showed a high level of mosaicism.

What is known already
Most women with TS experience a premature reduction or complete loss of fertility due to an accelerated loss of gametes. To determine whether fertility preservation in this group of patients is feasible, there is a strong need for information on the X chromosomal content of ovarian follicular and stromal cells.

Study design, size, duration
Small follicles (<50 µm) and stromal cells were isolated from ovarian tissue of young TS patients and analysed for their X chromosomal content. In addition to ovarian cells, several other cell types from the same patients were analysed.

Participants/materials, setting, methods
After unilateral ovariectomy, ovarian cortex tissue was obtained from 10 TS patients (aged 2–18 years) with numerical abnormalities of the X chromosome. Ovarian cortex fragments were prepared and cryopreserved. One fragment from each patient was thawed and enzymatically digested to obtain stromal cells and primordial/primary follicles. Stromal cells, granulosa cells and oocytes were analysed by FISH using an X chromosome-specific probe. Extra-ovarian cells (lymphocytes, buccal cells and urine cells) of the same patients were also analysed by FISH. Ovarian tissue used as control was obtained from individuals undergoing oophorectomy as part of their gender affirming surgery.

Main results and the role of chance
Ovarian follicles were detected in 5 of the 10 patients studied. A method was developed to determine the X chromosomal content of meiosis I arrested oocytes from small follicles. This revealed that 42 of the 46 oocytes (91%) that were analysed had a normal X chromosomal content. Granulosa cells were largely 45, X but showed different levels of X chromosome mosaicism between patients and between follicles of the same

patient. Despite the presence of a low percentage (10–45%) of 46, XX ovarian cortex stromal cells, normal macroscopic ovarian morphology was observed. The level of mosaicism in lymphocytes, buccal cells or urine- derived cells was not predictive for mosaicism in ovarian cells.

Limitations, reasons for caution

The results are based on a small number (n = 5) of TS patient samples but provide evidence that the majority of oocytes have a normal X chromosomal content and that follicles from the same patient can differ with respect to the level of mosaicism of their granulosa cells. The functional consequences of these observations require further investigation.

Wider implications of the findings

The results indicate that despite normal ovarian and follicular morphology, stromal cells and granulosa cells of small follicles in patients with TS may display a high level of mosaicism. Furthermore, the level of mosaicism in ovarian cells cannot be predicted from the analysis of extra-ovarian tissue. These findings should be considered by physicians when offering cryopreservation of ovarian tissue as an option for fertility preservation in young TS patients.

Study funding/competing interest(s)

Unconditional funding was received from Merck B.V. The Netherlands (Number A16-1395) and the foundation 'Radboud Oncologie Fonds' (Number KUN 00007682). The authors have no conflicts of interest.

Trial registration number

NCT03381300.

Introduction

Turner's syndrome (TS) is a heterogeneous genetic disorder in women with one intact X chromosome and complete or partial absence of the second X chromosome that affects 1 in every 2500 female live births [1, 2]. The presentation of TS is variable and includes typical phenotypic features, such as short stature, lymphedema, webbed neck, broad shield chest and congenital malformations of the heart and kidneys. The main concern for girls and women diagnosed with TS is the reduction or complete loss of fertility [3, 4]. Impaired fertility in TS is thought to arise mainly from accelerated germ cell apoptosis and impaired folliculogenesis during foetal life, leading to premature follicular depletion and gonadal dysgenesis [5, 6]. The degree of oocyte loss, however,

is variable, and the remaining function of the ovary after birth is thought to rely on the percentage of 46, XX cells in the ovaries [7]. In women from the general population, the number of gametes declines from approximately 2 million at birth to about 400 000 at the start of puberty [8]. In women with TS, the already- diminished oocyte pool at birth [5, 6, 9, 10] combined with this subsequent post-natal loss of gametes leads to ovarian insufficiency and incomplete sexual development during childhood in more than 80% of cases [11]. Several options exist to preserve fertility in women facing premature depletion of their gametes [12]. In adult women, these include freezing of ovarian tissue, mature oocytes or embryos, while in prepubertal patients ovarian tissue cryopreservation (OTC) is currently the only available option. When offering OTC to girls with TS who cannot wait until sufficient maturity to undergo oocyte cryopreservation, it should be considered at the earliest age possible in order to store as many primordial follicles as possible before their premature disappearance [12, 13]. Although OTC is widely and successfully used for fertility preservation/restoration in other patient groups [14-16], this does not guarantee that it will also be successful in TS patients. OTC is already routinely offered to TS women in several countries [13, 17-20], but there are no reports of children born from women with TS after auto-transplantation of their ovarian tissue [21]. The efficacy of OTC in this particular group of patients therefore remains to be confirmed. The success of OTC not only depends on the developmental competence of follicles that persist in the ovaries, but also on the ability of ovarian tissue from TS patients to support normal ovarian function. The functional capacity of the tissue after auto-transplantation might depend on the percentage of normal 46, XX cells that are required for follicular growth and ovulation. Aneuploidy in oocytes of primordial/primary follicles is also very likely to have functional consequences. In addition, genetic abnormalities in the layer of granulosa cells present in small (primordial and primary) follicles might impair normal follicular development [22, 23], as these cells not only control the arrest of the oocyte during the prophase of meiosis I, but also play a crucial role in normal follicular maturation [24]. Very little is known about the karyotype of these follicular cells in TS women. This information, however, is essential for understanding the mechanisms of premature follicular depletion and gonadal dysgenesis in this specific group of patients and, hence, to evaluate if OTC is a realistic option to preserve their fertility. As a first step toward the characterization of ovarian tissue from TS patients, we determined the X chromosomal content of oocytes and granulosa cells of small follicles, and stromal cells of ovarian cortex tissue in a cohort of 10 young TS patients with numerical abnormalities of the X chromosome. To this end we developed protocols to isolate intact small follicles from ovarian cortex tissue and efficiently separate the follicular cells, followed by fluorescence in situ hybridization (FISH) analysis of the X chromosome. In addition, a procedure was developed for determining the number of X chromosomal sister DNA strands in the normally tetraploid oocytes of meiosis I arrested follicles. The karyotype in peripheral

blood lymphocytes and cells of buccal smears and urine was determined to assess their predictive value for the karyotype of ovarian cells.

Materials and Methods

Patients

The current investigation is part of a nationwide trial 'Preservation of Ovarian Cortex Tissue in Girls with Turner Syndrome' (ClinicalTrials.gov Identifier: NCT03381300). Patients were recruited nationwide and referred to our tertiary fertility clinic between January 2018 and December 2018. Patients included in the current study are women with TS diagnosed with numerical X chromosome aberrations (45, X or 47, XXX), aged 2–18 years. Human ovarian tissue that was used as control was obtained from female-to-male transgender individuals undergoing oophorectomy as part of their gender affirming surgery.

Ethics

The study was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO NL57738.000.16). Written informed consent was obtained from all patients and/or their parents.

Collection of ovarian cortical tissue and estimation of the number of follicles per ovary

After unilateral ovariectomy, the ovary was collected in cold L15 medium (Lonza, Switzerland), immediately transferred to the laboratory and placed on a pre-cooled surface at 4°C. The medulla was removed from the cortex, after which cortex fragments of ~5 × 8 mm were prepared. Fragments were cryopreserved according to clinical standards [25]. For each patient, a single representative ovarian cortex fragment was available for research purposes. The fragment was thawed and cut in half. One-half was used for the isolation of small follicles and stromal cells (see below), the other half for histological analysis. The number of follicles was determined by complete serial sectioning (4 µm sections) of the tissue followed by haematoxylin–eosin (HE) staining of sections at 24 µm intervals. By examining stained sections at 24 µm intervals none of the primordial/primary follicles (follicle diameter ~45 µm) could have been missed. Since the follicular density in the ovaries of TS patients is low, counting the same follicle twice could be easily avoided and staining of all sections was therefore not necessary. In addition, in view of the heterogeneous distribution of follicles in the ovary, follicle density is expressed as the number of follicles per mm3 of cortex tissue.

Dissociation of ovarian cortex tissue

Individual small follicles and the stromal cells were isolated as described previously, with several modifications [26, 27]. To this end the tissue was cut into small pieces of ~1 × 1 × 1 mm and enzymatically digested in 4 ml of pre-warmed (37°C) L15 medium containing 0.1 mg/ml Liberase DH, 10 µg/ml DNase I (both from Roche diagnostics, Mannheim, Germany) and 1 mg/ml collagenase I from Clostridium histolyticum (Sigma life sciences, Israel) for 75 min at 37°C. The digestion mix was pipetted up and down every 15 min. The enzymatic reaction was stopped by the addition of 4 ml of cold L15 supplemented with 10% of foetal bovine serum (FBS; Life technology, Paisley, UK). The dissociated tissue was washed once with 8 ml of cold L15 medium by centrifugation at 500g and resuspended in 500 µl L15 medium. Next the cell suspension was transferred to a Petri dish and examined under a stereomicroscope. Small follicles (<50 µm) were manually picked up using a 75 µm plastic pipette (Research instruments, Falmouth, UK) and transferred to a droplet of L15 medium supplemented with 10% FBS at 4°C to prevent aggregation of follicles. To improve follicular cell spreading prior to FISH analysis, the follicles were treated with a solution of 0.06% trypsin, 1 mg/ml EDTA and 1 mg/ml glucose for 20 min at 37°C. Ovarian stromal cells were obtained from the cortex cell suspension, taking special care to avoid picking up any remaining follicles.

FISH analysis of lymphocytes, buccal cells, urine cells and ovarian cells from TS patients

FISH analysis of extra-ovarian cells was performed following standard protocols [28]. Ovarian follicles or stromal cells were transferred to 100 µl droplets of 0.04 mM KCl on a slide and incubated for 20 min at 37°C. Next, slides were allowed to dry and then pre-fixed in 300 µl of 0.05 mM KCl/7.5% acetic acid/22.5% methanol for 2 min at room temperature. Final fixation was performed by covering the slide with methanol/acetic acid (3:1) for 2 min at room temperature. FISH was performed according the manufacturer's instructions with chromosome X and chromosome 18-specific centromeric probes (CEP X (DXZ1) and CEP 18 (D18Z1); Vysis, Abbott, IL, USA). Fluorescent images were captured, and the signal(s) for the X chromosome was evaluated in somatic cells only when two signals of chromosome 18 were visible. In most oocytes, only one signal could be detected for each chromosome. Slides were counterstained with 4,6-Diamidino-2- Phenylindole (DAPI). The presence of different cell lines in the patients was determined by routine cytogenetic analysis of peripheral blood lymphocyte (30 cells), followed by FISH analysis of buccal cells (100 cells) and urine-derived cells (100 cells), following clinical standards.

Results

Determining the X chromosomal content of peripheral blood lymphocytes and cells from buccal smears and urine in patients with TS. From the study population five patients with a non-structural, mosaic karyotype 45, X / 46, XX (/ 47, XXX) and five patients with a 45, X monosomy were selected based on order of inclusion in the study (Table I). In the mosaic patients four out of five were of the 45, X / 46, XX karyotype (Patients B–E), while in Patient A three cell lines (45, X / 46, XX / 47, XXX) were found. The ratio between the different cell lines varied considerably between tissues of the same patient (**Figure 1**). For example, in Patient A the majority of lymphocytes had a 47, XXX karyotype while in urine comparable numbers of 45, X and 47, XXX cells were observed. In Patient E, the ratio between 45, X and 46, XX cells in lymphocytes and buccal cells was approximately 1, while in urine only 45, X cells were found without any detectable mosaicism.

Table I. Characteristics of the patients with Turner syndrome. In this study 10 patients with Turner syndrome were included.

Patient	Age (years)	Cell lines (lymphocytes and buccal cells)	Number of follicles per mm^3 tissue
A	8	45, X / 46, XX / 47, XXX	11
B	5	45, X / 46, XX	64
C	15	45, X / 46, XX	45
D	16	45, X / 46, XX	6
E	15	45, X / 46, XX	3
F	14	45, X	0
G	9	45, X	0
H	13	45, X	0
I	3	45, X	0
J	17	45, X	0

Macroscopic morphology of ovaries

Intact ovaries were obtained by laparoscopic unilateral ovariectomy and photographed before preparation of cortex fragments. As illustrated in **Figure 2**, the monosomy patients showed gonadal dysgenesis with bilateral streak ovaries. The ovaries of the five mosaic patients were of normal macroscopic morphology.

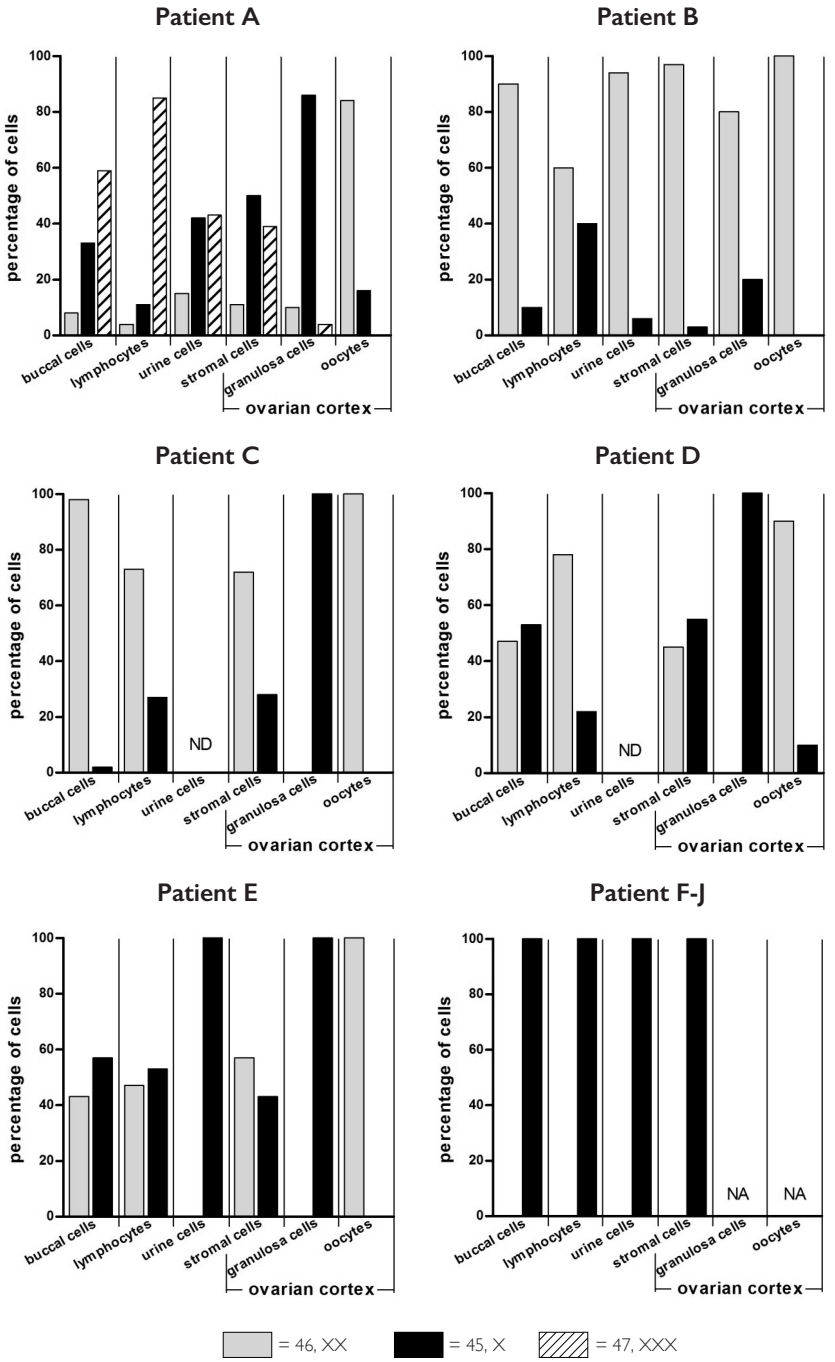
Patient A
Patient B
Patient C
Patient D
Patient E
Patient F-J
percentage of cells
100
80
60
40
20
0
buccal cells
lymphocytes
urine cells
stromal cells
granulosa cells
oocytes
ovarian cortex
ND
NA
= 46, XX
= 45, X
= 47, XXX

Figure 1. Mosaicism in extra-ovarian tissues and ovarian stromal cells, granulosa cells and oocytes from patients with Turner syndrome. In patients with Turner's syndrome (TS), lymphocytes (n =30), cells from buccal smears (n =100) and urine (n =100) were analysed by FISH with an X chromosomal peri-centromeric probe. The percentage of cells with a particular karyotype is indicated. In Patient A three different cell lines (45, X / 46, XX / 47, XXX) were detected, Patients B–E were mosaic for 45, X and 46, XX cell lines, while in Patients F–J only 45, X cells were found. In addition, ovarian cortex components including stromal cells (n = 100), granulosa cells (n ≥100) and oocytes were karyotyped. The number of oocytes was 19, 12, 2, 9 and 4 for Patients A, B, C, D and E, respectively. In Patients F–J no follicles were detected. ND=not done; NA= not applicable.

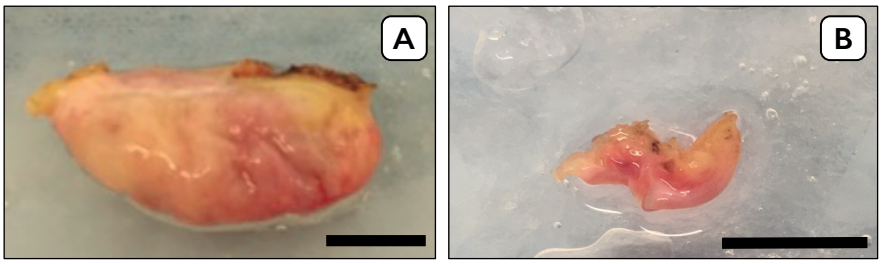

Figure 2. Macroscopic images of ovaries from TS patients. After unilateral ovariectomy intact ovaries were transported to the laboratory and photographed. A representative example of an ovary from a mosaic 45, X / 46, XX girl (Patient D) with normal morphology and volume is shown in panel A. The small fibrous streak ovary shown in photo B is from a girl with 45, X monosomy (Patient I). The brown discoloration of the tissue is due to electrocauterization during surgery. Bars represent 1 cm.

Designing a protocol for the separation of ovarian cortex cell components prior to FISH analysis

To determine the X chromosomal content of ovarian stromal cells, follicular granulosa cells and oocytes by FISH, we first designed a protocol to efficiently separate these ovarian cell components (**Figure 3**). To this end ovarian cortex fragments were prepared after unilateral ovariectomy. One part of the ovarian cortex fragment that was available for research purposes was used for histological analysis to determine the number of follicles (**Figure 3C and F**). The remaining part was enzymatically digested to yield a suspension consisting mainly of single stromal cells and, if present, individual follicles (**Figure 3D and G**). When no follicles were observed histologically, part of the stromal cells was used directly after the enzymatic digestion for FISH with centromere probes specific for the X chromosome and, as a control, chromosome 18. (**Figure 3E**). When small follicles (<50 µm) were present in the cell suspension these were picked

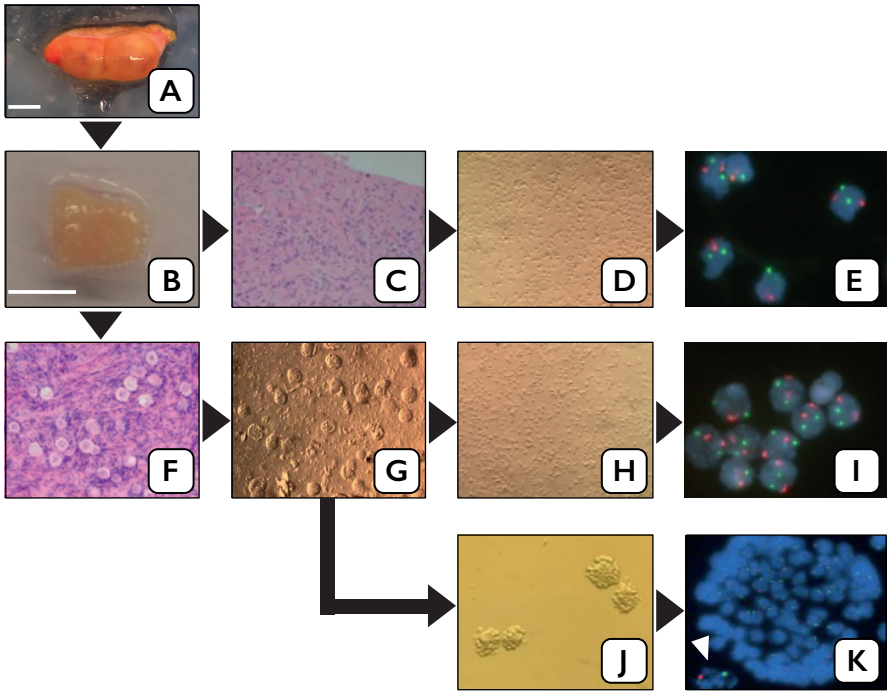

Figure 3. Flow scheme for separating ovarian cortex cellular constituents prior to FISH analysis. After the surgical removal of the intact ovary (A), cortical fragments were prepared. Part of one representative cortex fragment (B) was analysed by standard haematoxylin–eosin staining for the presence of follicles. When no follicles were present (C), the remaining part of the fragment was used to make a suspension of stromal cells (D), for interphase FISH with chromosome X (green) and chromosome 18 (red)-specific probes (E). When follicles were present (F), the remaining part of the cortex fragment was used to make a cell suspension (G) from which small follicles were manually picked up. These isolated follicles were subjected to further digestion (J) and subsequently analysed by FISH. The white arrowhead points to the signals from the oocyte (K). Part of the remaining cell suspension (H) was used for FISH analysis of stromal cells (I). Bars represent 1 cm (A and B) or 100 µm (C, D, F–H and J). Original magnification of FISH signals was ×630 (E, I and K).

up manually and analysed separately by FISH (**Figure 3K**). After follicle isolation, part of the stromal cells from the cell suspension (**Figure 3H**) was used for FISH analysis on separate slides (**Figure 3I**). Interpretation of the FISH signals of follicles that were isolated directly after the enzymatic digestion of the cortex tissue proved to be problematic, as nuclei of the granulosa cells of individual follicles were generally not sufficiently spaced to unambiguously determine the number of hybridization spots per

nucleus. In addition, the oocyte-specific FISH signals were frequently obscured by granulosa cell DNA (**Figure 4A and B**). To obtain more dispersed granulosa cell nuclei for FISH analysis we introduced an additional enzymatic treatment of isolated follicles with trypsin. This treatment visibly affected the follicles, showing partial rounding of granulosa cells on the surface of the follicle, but did not lead to detachment of cells from the follicle (**Figure 3J**). FISH analysis and DAPI counterstaining of these follicles clearly showed more dispersed granulosa cell nuclei and, more importantly, oocyte nuclei that were not covered by granulosa cell nuclei (**Figure 3K, 4C and D**).

Determining the karyotype of ovarian stromal cells

In the five patients (F–J) with 45, X monosomy in lymphocytes, buccal cells and urine cells, no follicles were found in the HE stained sections of ovarian cortex tissue, nor in the cell suspensions used for FISH (**Table 1, Figure 3C and D**). Karyotyping of the ovarian stromal cells of these patients showed exclusively 45, X cells (**Figure 1**). In the mosaic TS patients, the karyotype of the stromal cells showed a ratio of cell lines (45, X; 46, XX and 47, XXX) that was similar to that of cells from urine (Patients A and B), lymphocytes (Patients C and E) or buccal cells (Patients B, D and E).

Karyotyping granulosa cells from small ovarian follicles

The number of X chromosomes was determined in granulosa cells from small follicles isolated from ovarian tissue from the five mosaic TS patients (A–E). Although over 98% of the follicles in these tissues were either in the primordial or primary stages of development (**Figure 5A**), a small number of secondary follicles was observed as well (**Figure 5B**). After enzymatic digestion of the tissue and subsequent purification of the small follicles, the X chromosomal content of their granulosa cells was determined. Remarkably, in three out of five patients with a mosaic pattern all granulosa cells were 45, X (Patients C–E). In the other two mosaic patients, the granulosa cells displayed a mosaic karyotype with the majority being 45, X in Patient A, and 46, XX in Patient B (**Figure1**). In Patients B, D and E, efficient spreading of follicular cells allowed us to determine the number of X chromosomes in at least 22 granulosa cells from individual small follicles (**Figure 6**). The ratio of the 45, X and 46, XX granulosa cells varied considerably between follicles from the same ovary. In Patient B, a follicle was observed with exclusively 46, XX granulosa cells, while six other follicles of this patient showed a percentage of 45, X granulosa cells varying from 13% to 59%. The granulosa cells of 10 individual follicles of Patients D and 5 individual follicles of Patient E were all 45, X (**Figure 1 and 6**). Although a large number (45–72%) of the stromal cells of Patients C–E was 46, XX, we did not observe any cells with a 46, XX karyotype amongst the granulosa cells of these patients, strongly suggesting that stromal cells do not co-purify with the follicles. The co-purification of theca cells is also very unlikely since small follicles do not yet contain this cell type [29].

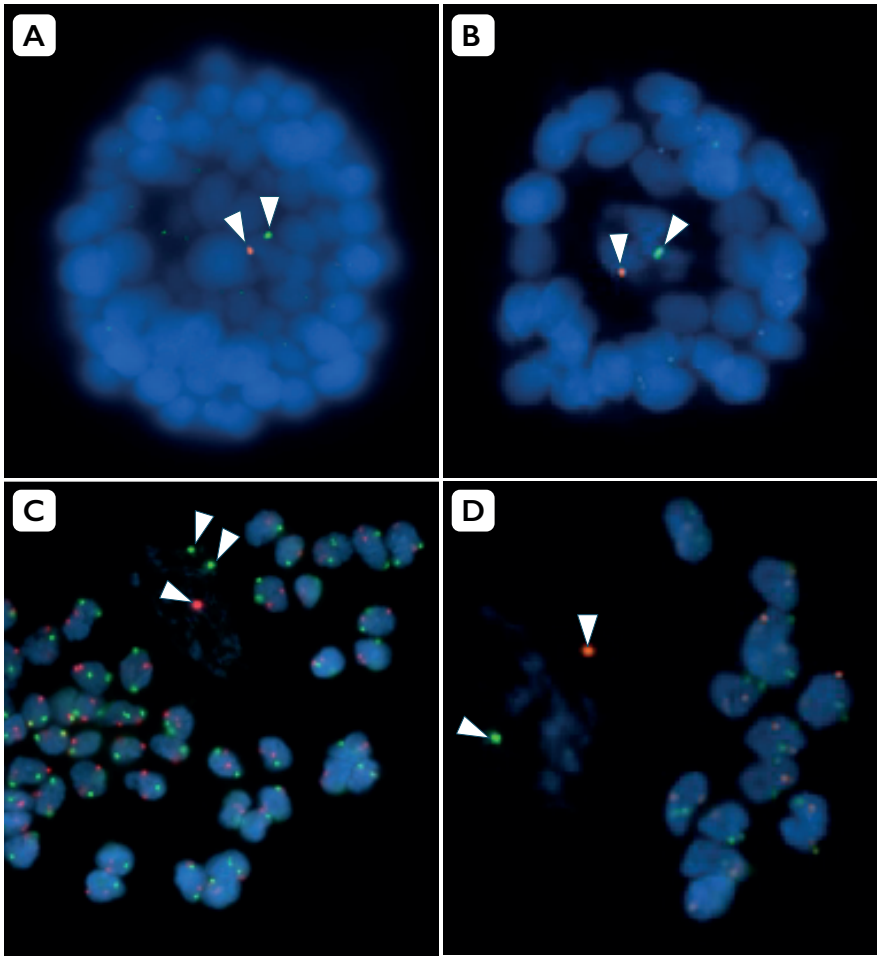

Figure 4. After treatment of isolated follicles with trypsin the follicular cells become more available for FISH. Cells of individual small follicles isolated from a suspension of ovarian cortex remain clumped during preparation for FISH, obscuring signals of individual granulosa cells and the oocyte (panels A and B). Treatment with trypsin of isolated follicles prior to FISH resulted in less cell clumping and allowed karyotyping of granulosa cells and oocyte of the same follicle (panels C and D). Note that the DAPI counterstain in the trypsin treated follicles reveals that the DNA of the oocyte is much more diffuse and can be easily distinguished from DNA of the granulosa cells. FISH signals for the X chromosome (green) and chromosome 18 (red) of the oocytes are indicated by arrowheads. Original magnification was ×630.

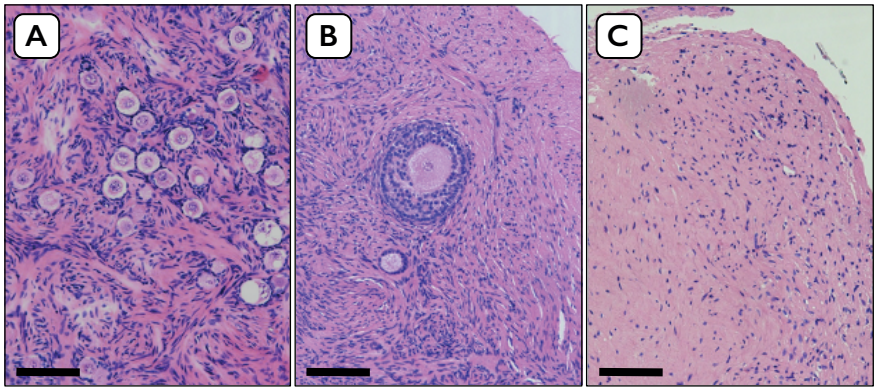

Figure 5. Histological sections of ovarian cortex from patients with TS. Haematoxylin–eosin stained 4-µm sections were prepared from cortical tissue from ovaries of mosaic (panels A and B) and 45, X monosomy TS patients (panel C). In addition to the variable number of small follicles in the tissue of the mosaic patients (panel A), a low number (<2%) of secondary follicles were observed (panel B). The ovarian tissue of the monosomic 45, X patients contained no follicles and showed a fibrous texture with relatively low number of cells (panel C). Bars represent 100 µm.

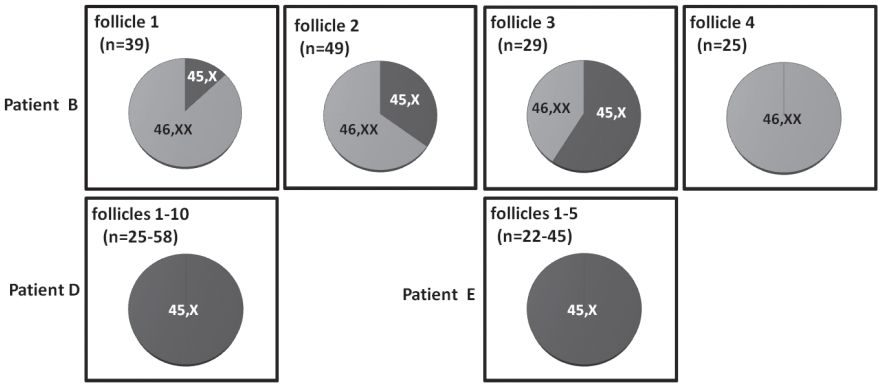

Figure 6. FISH analysis of granulosa cells from individual follicles. The ratio between 46, XX and 45, X granulosa cells from the same small follicle varied considerably. FISH analysis of six individual small follicles from Patient B revealed that the percentage of 45, X granulosa cells varied from 0% to 59% (top row; four follicles are shown). In Patients D (337 granulosa cells from 10 follicles) and E (152 granulosa cells from 5 follicles) all granulosa cells were 45, X. For Patients B and D at least 25 granulosa cells per follicle were analysed and for Patient E at least 22 granulosa cells per follicle.

Karyotyping the oocytes from small ovarian follicles

The FISH signals for the X chromosome and the control chromosome 18 were considerably more intense in the nuclei of oocytes than in the granulosa cells. Oocytes in primordial/primary follicles are arrested in the prophase of meiosis I and contain four copies of each chromosome held closely together by a synaptonemal complex [30]. This close proximity results in just a single but strong hybridization signal for the four clustered copies of each chromosome (**Figure 4 A, B and D**). In contrast to somatic cells it is therefore not possible to simply count the hybridization spots to determine

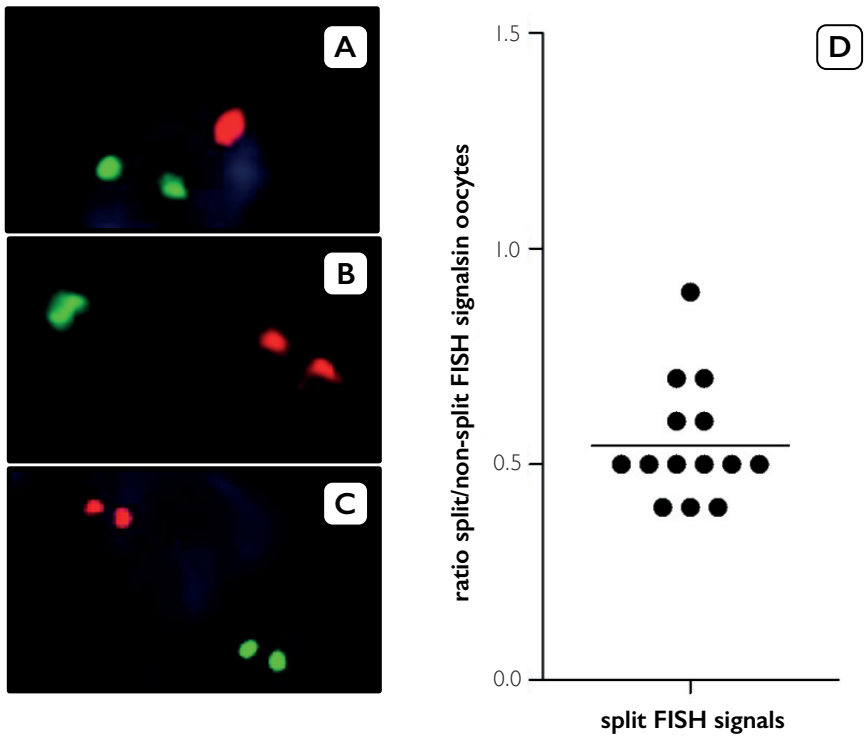

Figure 7. The surface of FISH signals can be used to karyotype oocytes from small follicles. To assess whether the surface of FISH signals can be used to determine the X chromosomal content of oocytes in the prophase of meiosis I, we measured the surface of split signals for the X chromosome (panel A), for chromosome 18 (panel B) or for both chromosomes (panel C) and compared these to the signal surface of the other chromosome. The surface ratio is shown in panel D and indicates that the surface of a split FISH signal is approximately 0.5 compared to the non-split signal of the other chromosome in the same oocyte. A 50% reduction in the number of X chromosomal sister chromatids will therefore lead to a reduction in the surface ratio between FISH signals for the X chromosome and chromosome 18.

the number of X chromosomes of a particular oocyte. Should an oocyte lack half of its X chromosomes it is likely that the corresponding FISH signal becomes less intense. To validate this assumption, we analysed the FISH results from a number of oocyte nuclei in which the hybridization signal for either the X chromosome or chromosome 18, or both, was split into two discrete spots (**Figure 7A–C**). By determining the surface of the hybridization spots as a measure for intensity, we found that the ratio of FISH signals between a single spot of a split signal and the control (non- split) signal in the same oocyte was approximately 0.6 (mean, 0.6; range 0.4–0.9; **Figure 7D**). Next we analysed the ratio of FISH signals for the X chromosome and chromosome 18 in the oocytes of small follicles from TS patients and two non-Turner 46, XX control patients (**Figure 8**). The mean of the ratio of the X chromosome/chromosome 18 FISH signals of individual oocytes was between 1.0 and 1.1, which was similar to the oocytes of the

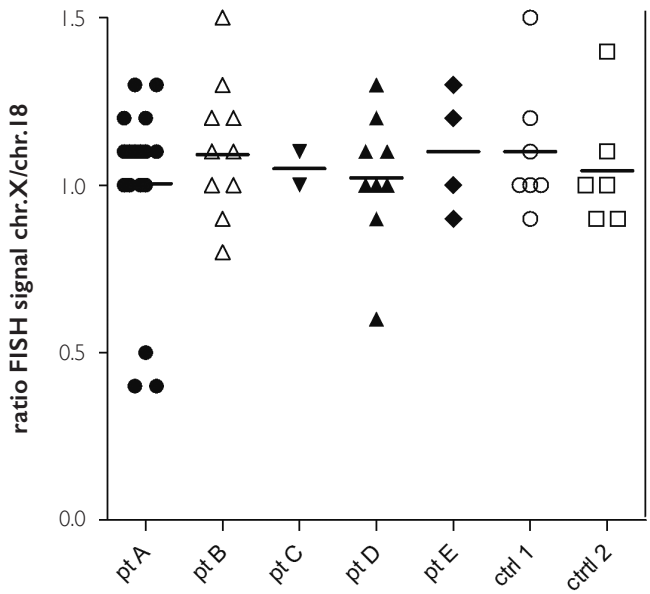

Figure 8. Most oocytes of small follicles from mosaic TS patients are 46, XX.
The ratio between FISH signals from the X chromosome and chromosome 18 was measured for 46 oocytes from 5 mosaic TS patients (pt) and 13 oocytes from 2 controls (ctrl). The ratio in the controls varied between 0.9 and 1.5. In TS Patients B, C and E, a similar distribution was seen, indicating that these oocytes had a normal X chromosomal content. In Patients A and D, the majority of the oocytes displayed a normal ratio, while in three oocytes from Patient A and one oocyte from Patient D, the ratio varied between 0.4 and 0.6, which is indicative of a reduction in the number of the X chromatids by half.

control patients. This indicates that most oocytes from the patients with a mosaic pattern had the normal number of four X chromosomal sister DNA strands. However, in Patients A and D the FISH signal ratio was found to be approximately 0.5 in 3 out of 19 oocytes and 1 out of 9 oocytes, respectively. This implies that in these oocytes probably only half of the normal X chromosomal DNA content was present.

Discussion

Fertility preservation by cryopreservation of ovarian cortex tissue is being performed on an experimental basis in young women with TS in several countries [13, 17-20]. However, very little is known about the genetics of ovarian cells in this group of patients, and this may well determine the final outcome of this fertility preservation procedure. In this study, we have therefore developed methods to analyse the X chromosomal content of both oocytes and granulosa cells from small (primordial and primary) follicles, and stromal cells from ovaries of young TS patients. Determining the number of chromosomes in diploid somatic cells is relatively straightforward. FISH with a centromere-specific probe will give two signals in each cell representing a pair of homologous chromosomes. However, analysis of oocytes from small follicles is more challenging as these oocytes are arrested in the prophase of meiosis I, and therefore tetraploid. At this stage of meiosis, each pair of homologous maternal and paternal chromosomes is held together by a synaptonemal complex [30]. The very close proximity of the homologous chromosomes in these complexes prevents the identification of individual chromosomes by FISH analysis. This unique organization of genetic material in the oocytes, as present in small follicles, explains why we observed only a single strong signal after hybridization with chromosome-specific probes, which was more intense compared to the signals in the nuclei of granulosa cells or stromal cells. Determining the karyotype of these oocytes is therefore not possible by simply counting the number of signals for each chromosome. Instead, the intensity of the FISH signal should be used for analysis, assuming that a reduction in number of a certain chromosome in the tetraploid oocyte will lead to a less intense FISH signal. To evaluate whether the number of sister DNA strands of a certain chromosome is indeed reflected by the intensity of the FISH signals in oocytes, we analysed images of oocytes of both TS patients and controls, in which the FISH signal for the X chromosome, chromosome 18 or both chromosomes was separated into two discrete spots. In these oocytes, the synaptonemal complex holding the four chromatids together was probably disrupted, resulting in not one (as in most oocytes) but two signals, each containing half of the genetic material. By using the surface area of the FISH spots as a measure for spot intensity, we found that intensity for each of these spots was indeed reduced by 50% and concluded that the surface area of FISH signals can be used to determine the

number of X chromosomal sister DNA strands in oocytes. There are several other techniques described for quantitative analysis of fluorescent intensity in microscopy images, such as epifluorescence imaging of FITC signals relative to their associated DAPI-stained signals [31], or by determining the ratio between two different FISH signals in the same cell [32]. However, most of the techniques suffer from lack of precision in the results and complexity of existing software and are therefore not straightforward for a researcher to use [33]. Furthermore, these techniques are optimized for cells during interphase or metaphase and have never been used to analyse the FISH signals of the tightly clustered chromosomes of a tetraploid human oocyte.

Karyotyping oocytes of TS patients from follicles at more advanced stages of maturation has been presented in a case report [19]. However, the oocytes analysed in that study already had demonstrated their capacity for development and might be part of a subset of gametes capable of progressing through meiosis and may therefore not be representative for the resting pool of small follicles in the ovary. The results of our oocyte FISH analysis seem reassuring; of the 46 oocytes from small follicles isolated from TS patients, 42 oocytes appeared to have a normal number of X chromosomes. Four oocytes with an 50% reduction of the FISH signal for the X chromosome were observed in two patients, suggesting that these oocytes were 45, X. In 45, X oocytes the X chromosome lacks a homologue and can therefore not achieve homologous synapsis during diplonema of prophase I. The finding of 45,X oocytes in humans is remarkable since this asynapsis is known to lead to oocyte elimination through meiotic silencing of unsynapsed chromatin [30]. Our findings may be explained by a process observed in mice, in which the 45,X oocytes evade elimination by non-homologous self-synapsis of the X chromosome [34]. The complete absence of follicles in ovarian tissue of the 45, X patients in our study argues against rescue of 45, X oocytes through self-synapsis. However, in contrast to the mosaic TS patients, the ovaries of the 45, X patients had a fibrous streak-morphology with a large amount of extracellular matrix that may not allow 45, X oocytes to develop or persist. Low numbers of follicles have been reported in ovaries of TS patients with 45,X monosomy, but karyotyping of the oocytes was not performed and cryptic mosaicism in these patients cannot be fully excluded [13]. Although most oocytes from TS patients we analysed had a normal X chromosomal content, this is not a guarantee that these follicles are truly functional. The human primordial/primary follicle is a multicellular structure in which the oocyte is surrounded by a single layer of granulosa cells. The oocyte depends on this layer of granulosa cells for its long-term arrest in meiosis I. During follicular growth there is extensive bidirectional signalling between the oocyte and its surrounding granulosa cells to ensure normal follicular development [24]. Our observation that all granulosa cells were 45, X in three of the five mosaic TS patients may have functional consequences for follicular development. In addition to follicles containing only 45, X granulosa cells,

we observed heterogeneity in the ratio of 45, X / 46, XX granulosa cells between different follicles from the same patient. This low-level or absence of mosaicism in granulosa cells that populate the small follicles suggests that these cells originate from a relatively small number of ovarian surface epithelium derived progenitor cells [35]. During follicle maturation the single layer of granulosa cells surrounding the oocyte in small follicles undergoes at least 10 mitotic divisions to produce the more than 2000 cells of the mature antral follicle [36]. As 45, X cells show an increase in apoptosis and have a prolonged cell cycle, the timing of expansion of the granulosa cell layer during maturation may be distorted [37-39]. The apparent histologically normal follicles populated with a high percentage of, or exclusively, 45, X granulosa cells could therefore be functionally impaired. Although the functional consequences of our observations require additional research, it could be possible that the mere presence of small follicles in the ovarian cortex tissue of TS patients does not guarantee normal ovarian function. No obvious correlation was found between the number of follicles in the ovary and the patient's karyotype of either the somatic cells or the oocytes. However, estimating the follicular density in a single piece of cortex should be interpreted with care in view of their uneven distribution throughout the cortex [40, 41]. In addition to the karyotype of the oocyte and the granulosa cells, the karyotype of the stromal cellular compartment may be of importance for the formation and persistence of a functional ovary as well. Stromal cells not only support the growing follicle physically and metabolically, but also provide the theca cells during follicular development [42, 43]. In the fibrous streak ovaries of the 45, X patients we found no evidence of mosaicism, which may well have contributed to their abnormal morphology. However, a certain degree of aneuploidy in the ovarian stromal cells does not seem to drastically influence the morphology of the ovary. Macroscopically, ovarian morphology was normal in the 45, X / 46, XX mosaic patients with 45–97% 46, XX stromal cells. Remarkably, normal ovarian macroscopic morphology was also found in a patient presenting a mosaic with only 10% 46, XX stromal cells. These findings are in line with previous published results. Ovarian stromal cells from a 45, X / 46, XX patient, from which normal 46, XX oocytes were retrieved after FSH stimulation, also showed 60% of 45, X cells [19]. This indicates that follicular development is possible even as the majority of the ovarian stromal cells are aneuploid. In this study, the analysis of extra-ovarian cells (lymphocytes, cells from buccal smears and urine) in TS patients with mosaicism does not seem to have any predictive value for the X chromosomal content of the ovarian cells (oocytes, granulosa and stromal cells). This substantiates the difference in levels of mosaicism, not only between patients but also between tissues, follicles and cells of the same type of a particular patient. Although the number of patients we investigated is relatively low, it is likely that the analysis of additional patients will only further support the notion that no two TS patients are the same with regard to the variation in mosaicism. In none of the extra-ovarian tissues of the 45, X patients did we find evidence of mosaicism. This

suggests that analysis of not only lymphocytes but also buccal and urine cells, all revealing a 45, X pattern, might be indicative of presence of a non-functional ovary. Over the years the presence of oocytes and even pregnancies in apparently 45, X women have been reported [44, 45]. However, the karyotyping methods in these cases were not clearly defined, and one can assume that these apparently 45, X women were in fact individuals with cryptic mosaicism [46, 47].

In this paper, we have confined our study to patients with numerical abnormalities of the X chromosome. Structural abnormalities (e.g. isochromosome X, ring chromosome X, X-deletions or translocations) are also frequently found in TS. Karyotyping cells of TS patients with structural abnormality of the second X chromosome is not possible with the probes we used, as these are directed at repetitive pericentromeric sequences and will not reveal chromosomal aberrations that leave these sequences intact. Structural aberrations of the X chromosome can be detected with probes against single copy sequences in somatic cells. However, due to the unique organization of the genetic material in meiosis I arrested oocytes, karyotyping with single copy probes is likely to be more difficult due to their less intense hybridization signal compared to the centromere-specific probes. In conclusion, we believe this to be the first report that presents a detailed analysis of the X chromosomal content of oocytes, granulosa cells and ovarian stromal cells from ovaries of young patients with TS. We show that the majority of oocytes from small follicles of mosaic TS patients are normal, but all or part of the granulosa cells of individual follicles may be aneuploid. Follicles derived from the same ovary can differ regarding the level of mosaicism of their granulosa cells. High-level mosaicism was found in ovarian stromal cells without deviant macroscopic morphology of the ovary. The level of mosaicism observed in lymphocytes, buccal cells or urine-derived cells did not correlate with that of ovarian cells. These new findings suggest that despite the presence of morphologically normal ovaries and follicles in mosaic TS patients, the aberrant karyotype of their granulosa cells and ovarian stromal compartment may limit their capacity to support fertility. As a consequence, caution should be taken when counselling TS patients and their parents about fertility preservation options, to avoid unrealistic expectations regarding the success rate of this treatment. Clearly more research is required to reveal the functional consequences of high-level monosomy in ovarian tissue cells. With these data, a better-informed decision can be made on whether OTC should be offered to girls and women with TS, and whether the procedure is able to restore fertility.

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7

Patient-specific aspect

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Expect the Unexpected: Hidden Ovarian Mosaicism and Macroscopically Perioperative Left-Right Differences of the Ovaries in a Patient with Classical Turner Syndrome.

Submitted

Abstract

One of the most reported positive prognostic factors for fertility in females with Turner syndrome is 46, XX mosaicism. However, routine genetic testing of lymphocytes, buccal cells or urine cells does not rule out the presence of a cryptic 46, XX cell line in the ovarian tissue, as the sex chromosome content may vary between different tissues from the same patient.

In this case report we show a hidden 46, XX cell line in the ovary and a perioperative left-right difference in ovarian morphology in a 13-year-old female who recently underwent laparoscopic surgery for ovarian tissue cryopreservation. She had been diagnosed with 45, X monosomy, or classical Turner syndrome at the age of 8 years old but presented with spontaneous pubertal development. Ovarian tissue could be cryopreserved successfully, but few follicles were found. One year after the unilateral ovariectomy, she continued to have regular periods of vaginal bleeding with normogonadotropic laboratory findings, except for unmeasurable AMH and inhibin-B concentrations.

These unusual findings might be of interest for clinicians involved in fertility preservation counselling for patients with Turner syndrome.

Keywords

Turner syndrome, fertility preservation, ovarian tissue cryopreservation, ovarian genetics, ovarian morphology

Introduction

One of the major concerns for young females with Turner syndrome (TS) is the increased risk of infertility [1, 2]. Advanced technology and international consensus now allows physicians to discuss fertility preservation (FP) options in this group of patients [3]. However, the most difficult aspect of the counselling is the variability and unpredictability in the current presentation and future progress of TS for each individual female [4].

TS is caused by the partial or complete absence of one of the sex chromosomes in some cell lines (i.e. mosaicism) or all cells. A reduction of the X chromosomal content may affect the cell metabolism and hence tissue functionality. Consequently, the presentation of TS includes a variety of phenotypic features [5].

In the ovarian tissue, TS leads to premature ovarian insufficiency with minimal chances of spontaneous conception [6]. In addition, the likelihood of healthy offspring is decreased as compared to the general population, because of the increased risk for miscarriage, and a slightly higher chance of giving birth to a child with a congenital malformation [6, 7].

It is assumed that patient's fertility, but also the efficacy of FP in females with TS is mostly depending on the percentage of ovarian cells with a 46, XX karyotype [8, 9]. Preferably, the karyotype of the ovarian cells should be taken into account when patients are counselled for FP, but in routine care the genetic testing is mostly limited to peripheral blood lymphocytes. In some cases, the karyotype analysis is extended by the analysis of buccal cells or urine cells. However, a recent study showed that the karyotype of extra-ovarian cells (e.g. lymphocytes, buccal cells or urine cells) in patients with TS is not representative for the karyotype of their ovarian cells [8]. This indicates that the percentage of normal 46, XX cells not only varies between patients, but also between different types of tissues from the same patient, between different cell types within the same tissue and even between cells of the same cell type [8, 10].

Case report

We report the case of a 13-year-old female with TS, who recently underwent laparoscopic surgery for ovarian tissue cryopreservation in our centre. She had been diagnosed with TS by the age of 8 years upon a diagnostic work-up for growth retardation. Chromosome analysis on peripheral blood lymphocytes revealed a 45, X monosomy, or classical TS karyotype. No other TS-associated problems were detected and growth hormone therapy was started. To exclude Y-chromosome-specific sequences, a FISH analysis was performed on buccal cells following the standard protocols [11]. Surprisingly, a 47, XXX [78] / 45, X [33] / 46, XX [7] karyotype was reported for these cells.

The first signs of spontaneous puberty, i.e. breast gland development (thelarche), were seen by the age of 10 years. Two years later she reported monthly periods of vaginal bleeding (5-7 days). Endocrinological investigation showed normal-slightly increased Follicle Stimulating Hormone (FSH) levels (6.1-9.5 E/l; ref 1.7–21.5 E/l), normal Oestradiol (E2) levels (74-100 pmol/l; ref 45–1461 pmol/l), normal Luteinizing Hormone (LH) levels (1.2-2.0 E/l; ref 1.0–95.6 E/l), and a low Anti-Müllerian hormone (AMH) level (0.2 ug/l; ref 0.56–8.4 µg/l).

By the age of 13 years, she was referred to the gynaecologist of our centre to discuss FP options together with her parents. At that moment, she was having irregular vaginal bleedings since a few months. As a regular part of counselling, serum hormone concentrations were determined, revealing an AMH level <0.1 ug/l (ref 0.56–8.4 µg/l), and an FSH level of 8.1 E/l (ref 1.7–21.5 E/l). LH & oestradiol were not determined. The uterus and left ovary could be visualized with transabdominal sonography. The uterus appeared to be anteverted, with a normal size for the patient's age and an endometrial thickness of 11 mm. The antral follicle count (AFC) in the left ovary was 4, and no cysts or free fluid was reported. After comprehensive counselling, the girl and her parents decided for ovarian tissue cryopreservation in an experimental setting (Trial registration number NCT03381300) with the aim to preserve her fertility. Although transabdominal sonography is not conclusive for the presence of ovaries, we discussed the plausible scenarios of an absent or streak ovary. Informed consent was obtained to remove the 'normal-looking' ovary in the case that an ovarian left-right difference or single ovary would become apparent during the laparoscopic intervention.

During laparoscopy, four months later, a normal sized uterus was seen and a difference in ovarian volume was confirmed with a macroscopically 'normal-looking' left ovary and a streak right ovary (**Figure 1**). The left ovary could be removed for FP purposes without any complications. The resected ovary measured 30*20*15 mm (**Figure 2-A**). Serum hormone concentrations were measured before the ovariectomy was performed and showed an elevated FSH serum concentration of 23 E/l (ref 1.7–21.5 E/l), normal LH level of 7.3 E/l (ref 1.0–95.6 E/l.), low AMH level 0.1 ug/l (ref 0.56–8.4 µg/l), and an E2 level and inhibin-B level under the laboratory detection level (i.e. < 18 pmol/l and <10 ng/l).

Directly after the unilateral ovariectomy, the ovary was collected in cold L15 medium (Lonza, Switzerland) and transferred to the laboratory where it was placed on a pre-cooled surface at 4°C. The medulla was removed from the cortex after which cortex fragments of approximately 8*8*1 mm were prepared. Fragments were cryopreserved according to the local protocol [12]. Ten ovarian cortex fragments of approximately 8*8*1mm could successfully be cryopreserved for FP purposes. According to the protocol, one representative fragment was kept aside, divided into two and cryopreserved for research purposes. After thawing, one part was used for histological analysis, and the other part for performing FISH analysis on ovarian cells as

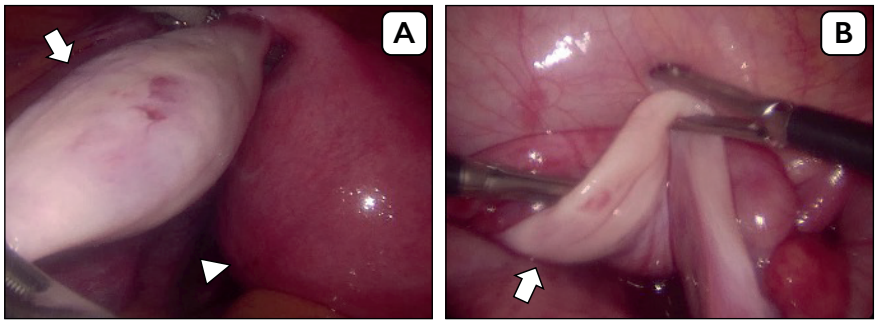

Fig. 1. Laparoscopic images of the genitalia interna. (A) Normal left ovary (arrow) and uterus (arrow head) (B) streak right ovary (arrow).

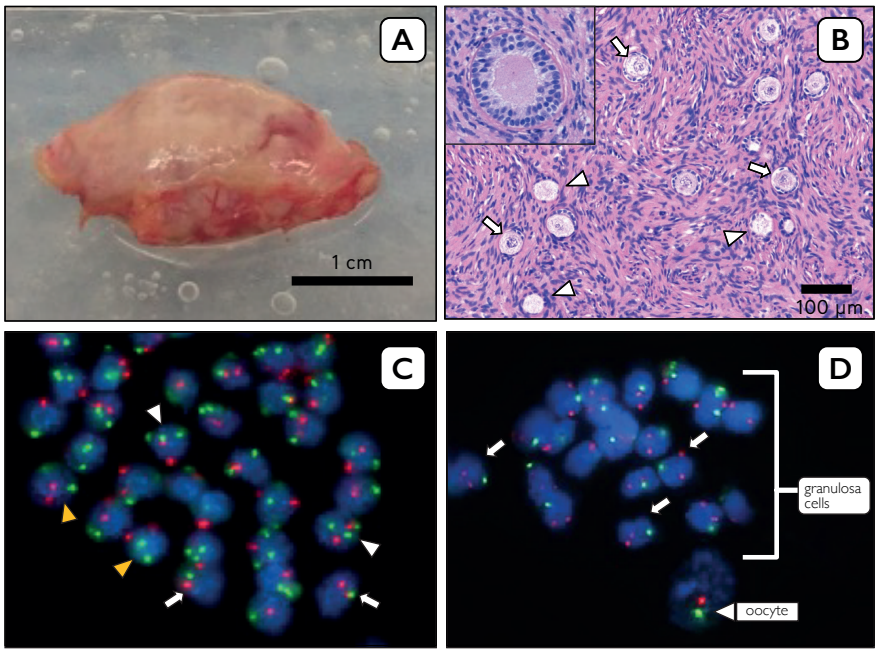

Fig. 2. Macroscopic, histologic and FISH analysis of ovarian tissue. (A) Macroscopically normal left ovary directly after unilateral ovariectomy. Length 3.0 cm Width 2.0 cm Height 1.5 cm. (B) Haematoxylin-eosin stained section of cortex tissue with empty follicles (arrows) and follicles with missing granulosa cells (arrow heads). The inset shows a multilaminar follicle with no recognizable oocyte. FISH analysis of cortex stromal cells (C) and of cells from a single follicle (D) with X-chromosome (green) and chromosome 18 (red) specific probes. Arrows point to 45, X cells, white arrow heads to 47, XX cells and yellow arrow heads to 46, XX cells. The FISH signals from the oocyte are indicated (D). Note that FISH signals are not all in the same focus plane.

described previously [8]. Although histological analysis revealed a normal ovarian morphology, no follicles were found in this fragment. The results were discussed with the patient and her parents during the check-up 2 months later, and they agreed to thawing 1-2 additional fragments for additional research.
In the first fragment, again, no follicles were found. The second fragment showed a decreased number of follicles with a follicular density of 7.0 follicles/mg tissue. Remarkably, several empty primordial follicles were seen, and even a multilaminar follicle without a recognizable oocyte. Furthermore, the tissue contained multiple primordial follicles with an incomplete layer of granulosa cells (**Figure 2-B**).
The second research fragment was enzymatically digested to yield a cell suspension consisting of stromal cells and primordial follicles. Stromal cells and individual primordial follicles were picked up manually and prepared for FISH analysis on separate slides. FISH analysis was performed with centromere probes specific for the X-chromosome, and a probe for chromosome 18 served as a control. The X-chromosome content of 80 stromal cells (**Figure 2-C**), 6 oocytes and 262 granulosa cells from 8 individual primordial follicles (**Figure 2-D**) could successfully be determined with FISH analysis. Karyotyping of the stromal cells (n=80) showed a mosaic pattern with 3 cell lines 45, X [68] / 47, XXX [18] / 46, XX [14], and a full-blown 45, X karyotype was seen in the granulosa cells (n=262) (**Figure 3**). Remarkably, all 6 oocytes that were analysed showed a normal (tetraploid 92, XXXX) karyotype (**Figure 3**).
In order to check if the X chromosome content of other cell types than lymphocytes or buccal cells was more representative for the karyotype of the ovarian cells, we also performed a FISH analysis on 100 urine cells according to the local protocol [8]. However, all cells that were investigated (n=100) showed a 45, X karyotype (**Figure 3**), and were thus, representative for the karyotype of the granulosa cells, but not for the oocytes and stromal cells in this patient.
The first year after surgery, the patient continued to have monthly vaginal bleeding periods of approximately 7 days. She had normal gonadotropic hormone levels (FSH 8.8 E/l; ref 1.7–21.5 E/ and, LH 2.3 E/l; ref 1.0–95.6 E/l) although E2 was relatively low (89 pmol/l, ref 45–1461 pmol/l.). AMH and Inhibin B levels remained unmeasurable. No other specific details were reported. Two years after surgery, the patient reported irregular blood loss (periods of six-day-long vaginal bleeding, every two months). Transabdominal sonography showed a normal uterine size for the patient's age and an endometrial thickness of 4 mm. The right ovary could not be visualized. Physical examination now showed complete breast development. Unfortunately, the serum hormone concentrations revealed hypergonadotropic hypogonadism with elevated FSH (84 E/l, ref 1.7–21.5 E/l) and LH (40 E/l, ref 1.0–95.6 E/l) levels, and an unmeasurable E2 concentration (<18 pmol/l). Therefore, oestrogen replacement therapy was started.

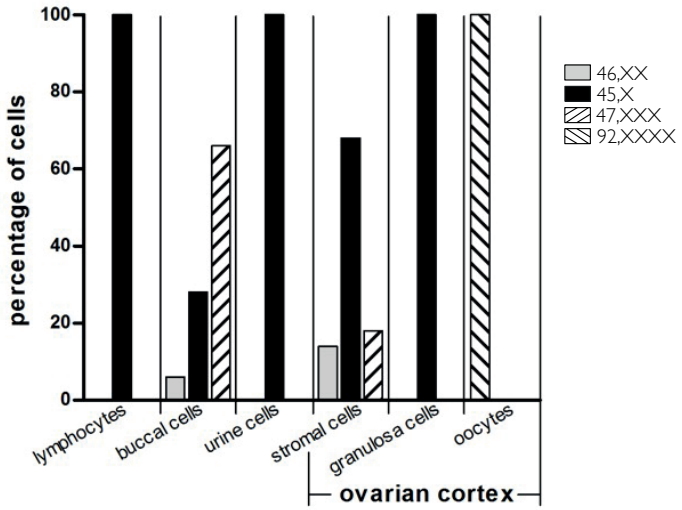

Figure 3. Mosaicism in extra-ovarian tissues and ovarian stromal cells, granulosa cells and oocytes. Lymphocytes (n=30), cells from buccal smears (n=100) and urine (n=100) were analysed by FISH with an X chromosomal peri-centromeric probe. The percentage of cells with a particular karyotype is indicated. In addition, the X-chromosomal content of ovarian cortex components including stromal cells (n=80), granulosa cells (n=262 from 8 individual unilaminar follicles) and oocytes (n=6) is shown. Three different karyotypes (45, X / 46, XX / 47, XXX) were detected in the somatic cells while all oocytes were tetraploid (92, XXXX).

Discussion

In this case report, we describe a second case [13] of a girl who had been diagnosed with classical monosomy TS (45, X), but presented with a hidden X mosaicism in her ovarian cells and a perioperative left-right difference in ovarian morphology during laparoscopic surgery. However, this is the first case reporting on the presence of both a streak ovary and normal ovary in a young female with TS. These unusual findings might be of interest for clinicians involved in FP counselling and TS care. Especially, since the efficacy of FP in this group of patients remains to be confirmed.

Our patient had been diagnosed with 45, X monosomy but presented with spontaneous monthly periods of vaginal bleeding by the age of 12. Ovarian tissue could be cryopreserved successfully, but a decreased follicular density was found as compared with the ovarian tissue of same-aged non-TS females undergoing FP [14]. One year after the unilateral ovariectomy, she continued to have regular periods of vaginal bleeding with normogonadotropic laboratory findings except for unmeasurable AMH and inhibin-B levels.

The karyotype of the non-ovarian cells (i.e. buccal cells, peripheral lymphocytes and urine cells) was somewhat representative for the stromal cells and granulosa cells but not for the karyotype of the oocytes that were found. Interestingly, all primordial follicles that were investigated, contained normal tetraploid 92, XXXX oocytes but were exclusively surrounded by diploid 45, X granulosa cells. Recently, similar findings in other females with TS have been reported [8, 13]. This might have functional consequences for the follicular development as the oocyte depends on the layer of granulosa cells for meiotic arrest but also for later stages of folliculogenesis [15]. However, in this patient, cryptic mosaicism in the oocytes and granulosa cells cannot be fully excluded because of the small number of primordial follicles (n=8) that was investigated.

The different macroscopic aspect of the ovaries may indicate a difference in X chromosome content between both ovaries. Although left–right differences in ovarian volume and antral follicle count have been reported before in healthy females of reproductive age and females with subfertility [13, 16, 17], there are no reports describing the presence of both a streak ovary and normal ovary in a young female with TS. We believe it is important to include the possibility of this rare finding during laparoscopic surgery, and how to deal with it, in the counselling for FP, even if we do not know yet what the clinical consequences are.

In this patient, the left-right difference in ovarian volume and aspect was already seen during transabdominal ultrasound (TUS). However, distinguishing precisely between anatomically normal ovaries and streak ovaries is not easy, and there might even be a chance that the ovaries cannot be visualized at all [18]. A Danish study showed that ovaries could be detected in 37% of young females with TS by TUS and in 55% by MRI ($P = 0{\cdot}1$) [19], but they did not confirm their findings with laparoscopic surgery, which is considered to be the gold standard.

In routine care, karyotyping of lymphocytes is the standard technique used to determine TS. In most centres, the diagnosis is based on 20-30 lymphocytes only, although it has been recommended to determine the karyotype of at least 50 metaphases to exclude <10% of mosaicism (CI 0.99) [20]. Interestingly, a recent cohort study in 142 adult patients with TS, revealed that the percentage of 45, X cells in lymphocytes and buccal cells was identical in less than one third of cases [21]. Therefore, these authors recommend adding buccal cell FISH analysis for a better global chromosomal evaluation, and, thus better care and follow-up.

Missing X chromosomal content in ovarian cells is likely to result in germ cell apoptosis and impaired folliculogenesis during foetal life [22, 23]. Therefore, one could assume that the described cases where follicles were found or pregnancies have occurred in females with 45,X monosomy are actually examples of undetected germ cell mosaicism [24, 25]. Unfortunately, key papers that are describing the presence or absence of follicles in females with TS [9, 26-29] generally do not report the methods used for karyotyping or the number of cells that were analysed.

Some clinicians are suggesting to make FP available for females with a mosaic karyotype only. For now, we believe it is too preliminary to close the doors for FP already for some subgroups with TS, purely based on the karyotype of 20-30 lymphocytes. We recommend other researchers within the field of TS and fertility to include a proper genetic analysis of different cell types and an imaging method, either US or MRI, to their research protocol, so that the predictive value of these diagnostic markers can be further explored and more knowledge regarding TS and fertility can be obtained.

Ethical approval

The study protocol for performing ovarian tissue cryopreservation and additional genetic testing in young females with TS has been approved by the Central Committee on Research Involving Human Subjects in November 2017 (CCMO NL57738.000.16). Written informed consent was obtained from the patient and her parents.

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8

General discussion

General discussion

In this thesis, the various challenges of ovarian tissue cryopreservation (OTC) in young females with Turner syndrome (TS) were explored. This thesis served as the groundwork for a long-term international cohort study (TurnerFertility trial, CCMO NL57738.000.16, ClinicalTrial ID NCT03381300) that started including the first patients in January 2018.
In this chapter the main findings are summarized, and discussed in a multidimensional approach, including the clinical implications and suggestions for future research. Furthermore, the importance of patient-involvement in medical research is pointed out and our research is positioned on Arnstein's [1] 'ladder of engagement' (**Figure 1**).

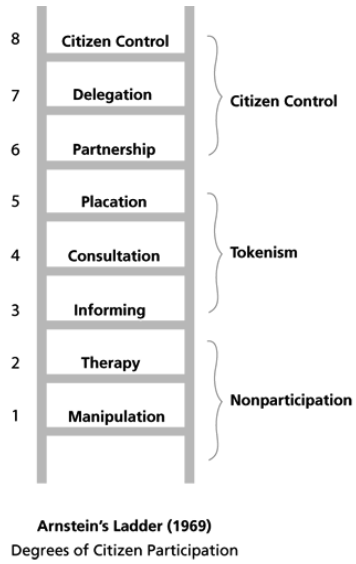

Figure 1. Arnstein's 'ladder of engagement'

Main findings and interpretation

1. *What is currently known about the presence of follicles in the ovaries of young females with TS? (**Chapter 2: In the literature**)*

For many years, it was thought that girls with TS were born infertile due to non-functional ovaries. However, scientists questioned this theory as increasing numbers of spontaneous pregnancies in females with TS were described. In 2002, a Swedish study group reported the presence of primordial follicles in the ovaries of 8 young females

with TS [2]. However, the number of follicles found was significantly lower as compared to the follicular density in unaffected girls undergoing laparoscopic ovarian biopsy. This new insight, combined with earlier research on TS foetuses [3-5], led to the hypothesis that a complete or partial loss of one sex chromosome in females causes an accelerated degeneration of germ cells, starting at the 13th week of foetal age. Due to this rapid loss, most females with TS reach menopause during childhood or adolescence. The timeline at which this occurs differs in every individual with TS [4].

II. *What is current state of art regarding (experimental) fertility preservation (FP) outcomes in females with TS? (**Chapter 2: In the literature**)*

Cryopreservation of mature oocytes (OC) and ovarian cortex tissue (OTC) has been performed experimentally in > 150 girls and adolescents with TS [4, 19, 27-37] [Clinicaltrial.org]. Thus far, there are no published records of girls with TS who have returned for autotransplantation or devitrification of oocytes and fertilization with embryo transfer. Hence, the efficacy of FP in females with TS is to date still unknown.
Since 2002, OTC has been performed experimentally in at least 83 young females with TS aged 6 – 19 years old [2, 6-9]. Ovarian tissue could be obtained in approximately 85% of the patients and no complications related to the surgical procedure have been reported. Primordial follicles were seen in the ovarian tissue of 43% of the cases. Positive predictive markers for the presence of follicles were mosaic TS, a spontaneous menarche, a spontaneous thelarche, a normal FSH level, and a normal AMH level.
Over the last ten years, eighteen cases of OC in females with TS have been reported [6, 8, 10-15]. The age of patients during the oocyte retrieval ranged from 13 to 28 years. On average, 11 oocytes (range 2 – 19) were retrieved per cycle, of which approximately 76% could be cryopreserved (range 69 – 100%). Remarkably, one patient developed a severe ovarian hyper stimulation syndrome with ascites. She fully recovered after abdominal paracentesis. The other females tolerated the procedure well, and no other complications were reported.

III. *Can we optimize the efficacy of OTC by using a non-invasive imaging technique to determine the density of follicles in human ovarian cortex fragments that are intended for fertility restoration? (**Chapter 3: Laboratory aspect**)*

In a proof of concept study, we investigated whether reflectance confocal microscopy (RCM) could be used as a non-invasive imaging technique to determine the vertical and horizontal distribution, and the number of follicles in human ovarian cortical tissue fragments intended for FP purposes. Our results show that RCM is a promising technique to determine the density of follicles ex vivo in living ovarian cortex fragments, apparently without compromising the vitality of the tissue. Safety studies and further optimization of the RCM technique with a focus on increasing the penetration depth are required before clinical use of RCM. After improvement, RCM could be applied to

evaluate the follicular density in ovarian tissue fragments of patients who are undergoing OTC. Ideally, ovarian tissue fragments with the highest number of primordial follicles could be selected for autotransplantation first. This is of clinical importance in all patients undergoing OTC, but especially in patients with a diminished ovarian reserve such as TS patients, because the effectiveness of OTC critically depends on the number of primordial follicles in the autograft.

IV. *Which ethical aspects should be considered regarding OTC in young females with TS? (**Chapter 4: Ethical aspect**)*

For some clinicians, performing OTC in females with TS remains a controversial topic. Therefore, we conducted an ethical Delphi study with international professionals and patient representatives to systematically discuss the advantages and disadvantages of OTC in females with TS. The aim of this study was to identify the most important ethical issues, formulate statements and reach group consensus.

In this international ethical Delphi study, patient representatives mainly highlighted arguments focusing on the psychological harm of infertility, whereas medical ethicists were more concerned about creating false hope resulting in psychological harm in the future. Gynaecologists and (paediatric-) endocrinologists underlined statements regarding non-maleficence ('do no harm').

After three rounds, the expert panel reached a consensus on eight key statements based on the most important ethical issues. The first two statements highlighted that infertility leads to psychological harm, and that patients with TS consider this their main concern (key statements 1 and 2). In addition, patients with TS should have equal access to FP options, in line with other patient groups (e.g. patients awaiting cancer treatments) (key statement 7). However, one should be aware of the increased risk of maternal morbidity and mortality associated with TS during a future pregnancy (key statements 5 and 6). Patients with TS should be counselled about the alternative options for future parenthood (i.e. adoption, fostering, and oocyte donation) (key statement 3). Furthermore, the option of voluntary childlessness should be discussed. All patients with TS interested in OTC should undergo psychosocial and cardiac screening and should be discussed by a multidisciplinary expert team (key statement 8). Great caution and restrictive (i.e. more negative, or even discouraging) counselling are recommended if laparoscopic surgery or pregnancy is contraindicated (i.e. in patients with severe cardiac comorbidity). Lastly, patients with TS should always be included in the consent process, regardless of their age (key statement 4).

V. *What is the standpoint of an international expert panel regarding OTC in young females with TS? (**Chapter 4: Ethical aspect**)*

Based on the set of 8 key statements that were mentioned above, our expert panel agreed that OTC in patients with TS should be offered, but only in a safe and controlled

research setting. This approval marks the first step in global acceptance of performing OTC in females with TS.

VI. *Which research should be performed to better inform TS patients about the feasibility and efficacy of OTC? (**Chapter 5: Clinical aspect**)*

We believe it is unethical but also logistically impossible to perform laparoscopic surgery followed by experimental OTC in minors in a randomized controlled trial research setting including randomization, blinding or treatment allocation. We suggest to perform a pilot study instead, i.e. an observational intervention study with long-term follow-up in a tertiary fertility clinic in the Netherlands including 100 females aged 2 through 18 years with classical Turner (i.e. 45, X monosomy) or Turner variants (i.e. 45, X mosaicism or structural anomalies, except Y-chromosomal material). The aim of this study is to investigate the occurrence of live birth in women with TS after OTC in childhood followed by auto transplantation in adulthood and to find reliable prognostic markers for estimating the ovarian reserve in girls with TS in the future.

Therefore, one fragment per patient is used to determine follicular density by conventional histology, and to perform FISH analysis of ovarian cells. Routine chromosome analysis is performed on both lymphocytes and buccal cells. A blood sample will be taken for hormonal analysis and all subjects will undergo a transabdominal ultrasound to determine the uterine and ovarian size. Patient characteristics, pregnancy rates, and pregnancy outcomes will be collected from the patient's medical record.

VII. *Is it possible to determine the X chromosomal content of various ovarian cells in young patients with TS? (**Chapter 6: Genetic aspect**)*

In an experimental laboratory study, we developed a method to analyse the X chromosomal content of oocytes and two supporting cell types (i.e. granulosa cells and stromal cells) in the ovarian tissue of young TS patients with fluorescence in situ hybridization (FISH) analysis. First, we developed a protocol to isolate intact small (primordial and primary) follicles and stromal cells from ovarian cortex tissue. A second protocol was developed to efficiently separate the follicular cells (i.e. the oocyte and surrounding layer of granulosa cells). **Figure 2** shows the three ovarian cell types that were analysed.

Determining the number of chromosomes in the diploid (i.e. containing two complete sets of chromosomes) somatic ovarian cells (i.e. granulosa cells and stromal cells) was relatively straightforward. FISH with a centromere-specific probe gave two signals in each cell representing a pair of homologous chromosomes. However, analysis of oocytes from small follicles was more challenging as these cells are arrested in the prophase of meiosis I, and therefore tetraploid (i.e. containing four complete sets of chromosomes). At this stage of meiosis, each pair of homologous maternal and paternal chromosomes is held together by a synaptonemal complex. The very close proximity

of the homologous chromosomes in these complexes prevents the identification of individual chromosomes by FISH analysis. Determining the karyotype of these oocytes is therefore not possible by simply counting the number of signals for each chromosome. Therefore, we developed an additional procedure for determining the X chromosomal content in the normally tetraploid oocytes of meiosis I arrested follicles, based on the intensity of the FISH signal.

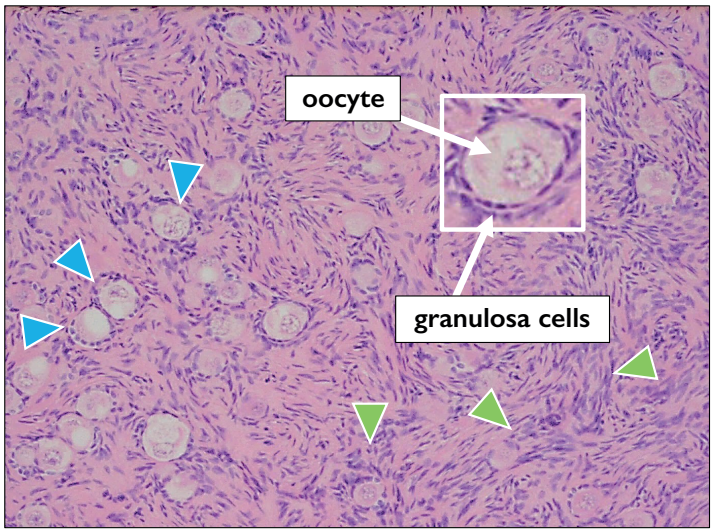

Figure 2. Haematoxylin and eosin stained section of human ovarian cortical tissue showing several small (primordial and primary) follicles (blue arrow heads) and stromal cells (green arrow heads). Each small follicle contains a single oocyte surrounded by a layer of granulosa cells (close-up).

VIII. *What is the X chromosomal content of the ovarian cells in young patients with TS? (**Chapter 6: Genetic aspect**)*

In addition, we performed a pilot study including the ovarian cortex fragments of ten young females with TS (mosaic TS n=5; classical TS n=5). Follicles were only found in the ovarian tissue of the five females with mosaic TS.

This 'proof of concept' revealed that in these TS patients, 42 of the 46 oocytes (91%) that were analysed had a normal X chromosomal content. Granulosa cells were largely 45, X but showed different levels of X chromosome mosaicism between patients and between follicles of the same patient. The karyotype of the stromal cells showed a ratio of cell lines (45, X; 46, XX and 47, XXX) that was somewhat similar to that of cells from urine, lymphocytes and/or buccal cells.

In the five patients with classical TS, no follicles were found, and the karyotyping of the ovarian stromal cells showed exclusively 45, X cells.

IX. *Is the X chromosomal content of the ovarian cells in young females with TS correlated with the X chromosomal content of other cell types? (**Chapter 6: Genetic aspect**)*

In the same pilot study, we showed that the analysis of extra-ovarian cells (lymphocytes, cells from buccal smears and urine) in TS patients with mosaicism does not seem to have any predictive value for the X chromosomal content of the ovarian cells (oocytes, granulosa and stromal cells). This substantiates the difference in levels of mosaicism, not only between patients but also between tissues and cells of the same type of a particular patient.

X. Is FP also feasible in patients with 45, X monosomy*? (**Chapter 7: Patient-specific aspect**)*

Some clinicians are suggesting making FP available for females with a mosaic TS only. For now, we believe it is too preliminary to close the doors for FP already for other TS genotypes, purely based on the karyotype of 20-30 lymphocytes.

In a case report, we report on a 13-year-old female who had been diagnosed with 45, X monosomy, or classical TS at the age of 8 years old but presented with spontaneous pubertal development. Ovarian tissue could be cryopreserved successfully, and follicles were found. This case of hidden ovarian mosaicism in a patient diagnosed with classical TS is not unique. Over the years the presence of oocytes and even pregnancies in several (apparently) 45, X women have been reported. However, the karyotyping methods in these cases were not clearly defined, and one can assume that these apparently 45, X women were in fact individuals with cryptic mosaicism.

As a clinician

As a clinician, I have experienced that humans are curious and always seeking for new technologies to improve their life. As clinicians, we bear the responsibility of practicing medicine to the best of our ability, where transparency to our patients is one of the most fundamental factors. If a medical technology is proven to be successful for one patient group (such as OTC in young cancer patients), it is logical that other patient groups such as those with TS also search for possibilities to benefit from this technique. However, the effectiveness of OTC in females with TS should be confirmed first, before it can be offered in daily practice. Nonetheless, OTC procedures have been performed in single TS patients without evidence of benefit and most of the time, without the insurance covering the costs of the surgical intervention and additional cryopreservation procedure.

I believe, just like our TurnerFertility expert panel [16], that it is better to offer OTC to females with TS in a safe and controlled research setting first. Most preferably, in a university medical centre with high experience in FP techniques and a dedicated team of counsellors and surgeons.
I am proud that with this research project, specialized consultation hours at Radboud university medical centre were launched. We are now one of the first centres in the Netherlands where Dutch patients with TS and their parents are structurally and fully counselled about fertility, FP and alternative options to become a parent in the future. I believe it is extremely important that patients are informed about this as soon as possible, following their diagnosis. The consultation should be patient-specific and age-specific and can be extended with additional counselling sessions in the future.
I really hope that this will lead to less psychological burden and thus, better health care, because patients are now better informed.
Qualitative research, including interviews and focus groups with patients and other stake holders, and health care evaluations could improve future counselling. Furthermore, our 'static' option grid should be further optimized to a real decision aid. In the future, a prediction model could help me and my colleagues to specify the counselling. In the meanwhile, I recommend other clinicians to add at least a buccal cell FISH analysis in patients diagnosed with 45, X monosomy or low-grade mosaicism. In most centres, karyotyping of 20-30 lymphocytes is the standard technique used to determine TS. We believe that in these particular patients, the analysis of 1) 2 different cell types, and 2) more cells, will result in a higher chance of identifying additional cell lines and thus, a better chromosomal evaluation.
Lastly, I hope that this thesis will generate more knowledge about TS in the general population, and lead to more equality and justice for TS patients regarding FP options. It would be grateful if in twenty years, when I am still working as a clinician, this thesis has led to a cascade of research, new insights regarding the early loss of the ovarian reserve in females with TS, and novel options to preserve the fertility in this specific group of patients.

As a researcher

As a researcher, I realize that we are still standing at the beginning of a long road. At this moment, FP in females with TS is still theoretical. This means, that we do not know yet if the cryopreserved ovarian tissue of females with TS is functional, and if autotransplantation in the future will lead to healthy offspring. In the worst-case scenario, we will perform a long-term intervention study in 100 young females, and none of them will profit from it.

This sounds controversial, especially as good clinical practice (GCP) is based on *primum non nocere* ('first, do no harm'), but we should also not forget the importance of the 3 other basic principles *autonomy* (what do patients want?), *beneficence* (how can we help these patients the best?) and *justice* (equal access to medical treatment options for all patients worldwide).

We should realize that only a few years ago our Oncofertility colleagues were standing at the same crossing. It takes imagination and dedication to introduce new treatment options, but also some courage and patience. You should be prepared for lots of criticism, and in the first few years, you will not have clear answers yet.

To ensure the safety of participants and the validity and integrity of study data, a data and safety monitoring board (DSMB), data monitor and an independent confidential advisor were appointed. Our research protocol underwent a lengthy process to become ethically approved. This long waiting time, we used to publish an overview of the existing literature regarding OTC in females with TS [17], to optimize the logistical processes and to improve the laboratory aspects of OTC, specifically for females with TS [18, 19]. Ethical approval was obtained by the end of November 2017, and thanks to our preparations we could start including the first patients January 2018. This long-term follow-up study will focus on the efficacy of OTC in females with TS including pregnancy rates, pregnancy outcomes and maternal, as well as foetal risks.

As it will take years for the final results of the main project (long-term follow-up study) to be expected, in vitro and in vivo studies could be performed in the meantime. These studies should focus on oocyte genetics, activation of immature oocytes in isolated ovarian tissue [20-23], maturation of immature oocytes obtained from cryopreserved ovarian tissue (IVM) [24], and the development of artificial gametes from stem cells [25] to optimize the chances for females with TS of becoming biological parents. Furthermore, qualitative research should focus on the optimization of counselling and the development of a decision aid. In cooperation with our team of paediatric surgeons, retrospective data regarding the surgical outcomes and the recovery of OTC in females with TS should be collected, evaluated and reported.

In case that OTC has proven to be a successful technique to preserve the fertility of (some) females with TS, it is essential to avoid unnecessary surgical interventions in the future. Ideally, a reliable prognostic model for estimating the ovarian reserve in females with TS as early as possible in life should be developed. The data of the TurnerFertility trial can be used for the development of this prognostic model but should also be validated in an external cohort. This model can be expanded with the data of an additional cohort study of our research group, i.e. the Minipuberty study, in which we focus on early indicators of exhaustion of the ovarian reserve in young children with TS. The effectiveness of other FP techniques in females with TS, such as vitrification of mature oocytes, should also be studied and reported. Furthermore, more information regarding the pros and cons of (intra-familiar) oocyte donation in females with TS

should be gathered and compared with the pros and cons of FP techniques.
Lastly, I would recommend future researchers to include a proper genetic analysis (i.e. the cell type(s) used for chromosome analysis and the number of cells that were determined), when reporting on TS and fertility outcomes. This important information is currently lacking in most published papers.

Patient and Public Involvement

As a researcher with little experience, I am proud to have contributed to the establishment of a new research theme (TurnerFertility) at the Radboud university medical centre. I believe we performed our research in a deliberate and thoughtful manner, especially since we actively involved patients in the research process. In 2014, the Lancet published the series *Research: increasing value, reducing waste*. Chalmers and his colleagues [26] covered the issue of how to decide which future research questions need answering, suggesting a few research approaches, particularly patient engagement in medical research. This thesis is an example of patient-initiated research as there was an increasing demand from patients and their parents to explore the possibilities of (experimental) FP options in girls and young females with TS. In 2016, the TurnerFertility research project started with a brainstorm meeting with patients and patient representatives in collaboration with the Dutch patient organization (Turner Contact Nederland). Afterwards, the research protocol [27] and patient information brochures were developed together with patients and patient representatives. Furthermore, patients and patient representatives were actively involved in the ethical approval and ethical Delphi study. Interim results were published in plain language on our website and in a newsletter, so that patients and their parents were well informed. Furthermore, regular information evenings were held to inform newly diagnosed patients and their parents about our research projects. Lastly, surveys were sent out and focus groups with patients and patient representatives were organized to evaluate our research, to discuss experiences and to share ideas about future projects.
Arnstein's [1] 'ladder of engagement' is commonly used by both patients and researchers to describe or explain the level of patient engagement in medical research (**Figure 1**). I hope that our research project can be positioned on one of the upper four rungs (Placation, Partnership, Delegated Power, Citizen Control). However, to move from tokenism to patient empowerment, the patient involvement in our research project should be further progressed by overcoming the barriers and enablers. For example as suggested by Ocloo *et al.* [28], by involving a more diverse group of patients and by making our meetings more attractive, inclusive and enjoyable, or by using different methods of involvement that go beyond traditional methods (i.e. meetings and surveys) such as supportive activities and informal venues. Furthermore, our research team should be easily accessible, and we could focus more on speaking slowly, avoid jargon, and communicate in other ways than speech / printed material (our E-mail box,

information letters and brochures), e.g. an interactive website including multimedia and visual materials.
Finally, I hope that in the future, more patients will be involved in the design and evaluation of medical research projects, because patient-involvement will contribute to a fresh perspective on research and meaningful healthcare improvements [26].

As a policy maker

The WHO states that all individuals have the right to decide freely the number, spacing and timing of their children and that patients should have access to all medical treatments [29]. Some people believe that FP services should be available for everybody, without discrimination. A few years ago, this has led to a debate to expand FP options to other patient groups (e.g. transgenders and girls with TS) than girls undergoing gonadotoxic cancer treatments. After ethical approval was obtained to perform FP in Dutch trans-males, it was expectable that also girls with TS would be a patient group demanding for the possibility to undergo FP in a research setting. However, expanding FP to other patient groups might raise some ethical concerns especially in females with TS, as its efficacy is still unknown, while performing surgery in very young children is required. In such complex situations, the Delphi method is used for decision-making. As stated in this thesis, an international expert panel of gynaecologists, (paediatric) endocrinologists, (medical) ethicists, patients and patient representatives stated that OTC should be offered to females with TS, but not yet in routine care. The efficacy should first be studied in a safe and controlled research setting. As a policy maker, I would initiate an international register, containing clinical information of all cases of FP in females with TS, including details on fertility outcomes and predictive markers. Secondly, further steps should be made in order to find a balance between good quality care and the economic burden to the society. These cost-benefit analyses should also focus on the psychological burden of infertility in females with TS, alternative options for biologically own children (e.g. oocyte donation or adoption) and the costs associated with this.

As a female

Unfortunately, I have met several adult TS patients who were not informed about their infertility or had received outdated information.
I believe that all humans would like to be well-informed about their body, health and reproductive system. This information should be age-specific and up to date. General information about the reproductive system can be given by parents and schoolteachers.

Most preferably it is provided from a young age onwards, frequently repeated, and extended with additional age-specific information at the appropriate moment.
In girls and females that have been recently diagnosed with TS, the specific information regarding TS and fertility should be provided by a dedicated team of caregivers. It is important that the girl and her parents receive this information as soon as possible after the diagnosis. Especially, since infertility, but also the uncertainty about their fertility is one of the major concerns for TS patients and their parents [30, 31]. This information should mainly focus on the pathogenesis of the early loss of germ cells or ovarian reserve, and ideally be extended with a patient-specific risk profile based on the best available evidence. Furthermore, the alternative options to become a parent should be discussed, such as foster ship, adoption or (intrafamilial) oocyte donation. Lastly, information should be given about TS and pregnancy, especially in high-risk patients. After all these issues have been addressed, FP can be suggested including the possible benefits and risks of the procedure.
Depending on the girl's personal situation, her age, and the communication with her parents, additional counselling from a paediatrician or a specialized paediatric psychologist could be considered. Furthermore, it could be helpful if she receives the information in different ways (i.e. presentation, personal counselling, brochures, internet, images) from different perspectives (i.e. paediatrician, gynaecologist, paediatric surgeon, paediatric psychologist, and/or peers).

Final conlusion

OTC could be a promising method to preserve fertility in females with TS. However, the efficacy of OTC in young females with TS is at present theoretical. Therefore, we suggest that OTC in females with TS is offered solely in a safe and controlled research setting. The TurnerFertility trial (CCMO NL57738.000.16, ClinicalTrial ID NCT03381300) emerged from the preparations that were made in this thesis. In this long-term follow-up study, we will focus on the efficacy of OTC in females with TS including pregnancy rates, pregnancy outcomes and maternal, as well as the foetal risks. Hence, it will take years for the final results to become available. In the meanwhile, my colleagues will focus on gathering more knowledge regarding the factors and pathways associated with the increased loss of germ cells in females with TS. In the future, targeted interventions could be developed to increase the chances for females with TS of becoming biological parents. In addition, qualitative studies are needed to improve the current health care, optimize the counselling process, prevent decision-regrets and psychological burden of counselling.

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9

Summary | Samenvatting

Summary

In this thesis, the various challenges of ovarian tissue cryopreservation (OTC) as a fertility preservation procedure for girls and young females with Turner syndrome (TS) were explored.

In **Chapter 2** we show that experimental fertility preservation (FP) procedures such as OTC and the vitrification of mature oocytes (OC) have been reported in more than 150 girls and adolescents with TS, but that the efficacy is still unknown due to the lack of follow-up data.

In young cancer patients, OTC is already an established fertility preservation method. Autotransplantation of cryopreserved ovarian tissue in cancer survivors has resulted in restoration of ovarian function, pregnancies and more than 130 live-births. The efficacy of ovarian function restoration is highly related to the number of follicles in the autograft. So preferably, the ovarian fragments with the highest number of follicles should be selected for autotransplantation purposes. This is of clinical importance in all patients undergoing autotransplantation of cryopreserved ovarian tissue, but especially in patients with a reduced ovarian reserve during OTC such as TS patients. However, determining the follicular density with conventional histology renders the tissue unsuitable for subsequent transplantation. In addition, follicles are not evenly distributed across the ovarian cortex surface, and therefore determining follicle density in one tissue fragment does not necessarily reflect the number of follicles in adjacent fragments. Ideally, a non-invasive imaging technique is used to determine the number of follicles in each ovarian cortex fragment, so that the fragments with the highest follicle density can be selected for autotransplantation first.

In **Chapter 3** we investigated whether reflectance confocal microscopy (RCM) could be used as a non-invasive imaging technique to determine the number of follicles, and the vertical and horizontal distribution, in human ovarian cortical tissue fragments. In this proof of concept study, we show that RCM is a promising technique to determine the density of follicles *ex vivo* in ovarian cortex fragments, apparently without compromising the vitality of the tissue. Safety studies and further optimization of the RCM technique with a focus on increasing the penetration depth in ovarian tissue are required before RCM can be applied in routine care.

Expanding OTC to girls and young females with TS raises complicated medical and ethical questions, and therefore remains a controversial topic for many clinicians. However, patient organizations are optimistic and demand equal access to FP options worldwide. In **Chapter 4** we explored the opinion of an international panel including

both professionals and patient representatives whether OTC should be offered to girls and young females with TS, or not. In a three-stage ethical Delphi study, the advantages and disadvantages of OTC in females with TS were systematically discussed. The aim of this study was to reach group consensus and to form an internationally accepted standpoint regarding OTC in girls and young females with TS. Our expert panel agreed that OTC should be offered to patients with TS, but only in a safe and controlled research setting. This approval marks the first step towards the global acceptance of performing OTC in females with TS.

A randomized controlled trial (RCT) is considered as the best research strategy when testing the effectiveness of new treatments. However, in our opinion, it is unethical but also logistically impossible to perform laparoscopic surgery followed by experimental OTC in minors in a randomized controlled trial research setting including blinding and treatment allocation. In **Chapter 5**, we suggest to perform a pilot study instead, i.e. an observational intervention study with long-term follow-up in a tertiary fertility clinic in the Netherlands including 100 females aged 2 through 18 years with classical Turner (i.e. 45, X monosomy) or Turner variants (i.e. 45, X mosaicism or structural anomalies, except the presence of Y-chromosomal material). The aim of this study is to investigate the occurrence of live birth in women with TS after OTC in childhood followed by autotransplantation in adulthood. In addition, the study results can be used to find prognostic markers for the presence or absence of follicles, so that ovarian reserve in girls with TS can be estimated better in the future, and/or at a young age.

If follicles are found in the ovarian tissue of a female with TS, the question arises if these follicles are truly functional, i.e. if they will lead to pregnancies and (healthy) offspring in the future. As the tissue functionality in TS appears to be largely dependent on the karyotype of the cells, we developed a method (**Chapter 6**) to analyse the X chromosomal content of oocytes and two supporting cell types (i.e. granulosa cells and stromal cells) in human ovarian tissue with fluorescence in situ hybridization (FISH). In addition, we performed a pilot study including the ovarian cortex fragments of ten young females with TS.

In the five patients with mosaic TS, a total number of 46 oocytes could be analyses, and 42 (91%) had a normal X chromosomal content. Surprisingly, the surrounding granulosa cells were largely 45, X but showed different levels of X chromosome mosaicism between patients and between follicles of the same patient. The karyotype of the stromal cells showed a variety of cell lines (45, X; 46, XX and 47, XXX) that was somewhat similar to that of cells from urine, lymphocytes and/or buccal cells. This substantiates the difference in levels of mosaicism, not only between patients but also between tissues and cells of the same type of a particular patient. In the five patients

with classical TS, no follicles were found, and the karyotyping of the ovarian stromal cells showed exclusively 45, X cells.

The chance that follicles are found in a 45, X monosomy patient is relatively small according to previous research. Some clinicians therefore suggest making OTC available for females with a mosaic TS only. For now, we believe it is too preliminary to close the doors already for some TS subtypes, purely based on the karyotype of 20-30 lymphocytes. To illustrate this, we report on a case (**Chapter 7**) of cryptic mosaicism in a 13-year-old female who had been diagnosed with 45, X monosomy, or classical TS at the age of 8 years old. She presented with spontaneous pubertal development, and recently underwent laparoscopic surgery for OTC. Ovarian tissue could be cryopreserved successfully, and follicles were found. All primordial follicles that were investigated, contained oocytes with a normal tetraploid 92, XXXX karyotype but were surrounded exclusively by diploid 45, X granulosa cells. Karyotyping of the stromal cells showed a mosaic pattern with 3 cell lines 45, X / 47, XXX / 46, XX, indicating the presence of additional cell lines besides 45, X.

In conclusion, OTC is a promising method to preserve fertility in females with TS. However, the efficacy of OTC in young females with TS is at present hypothetical. Therefore, we suggest that OTC in patients with TS is offered solely in a safe and controlled research setting. The TurnerFertility trial (CCMO NL57738.000.16, ClinicalTrial ID NCT03381300) emerged from the preparations that were made in this thesis.

Samenvatting

In dit proefschrift lichten we vanuit verschillende perspectieven toe welke uitdagingen er komen kijken bij het invriezen van ovariumweefsel (OTC) ten behoeve van fertiliteitspreservatie bij meisjes en jonge vrouwen met het syndroom van Turner (TS).

In **Hoofdstuk 2** beschrijven we dat bij meer dan 150 meisjes en jonge vrouwen met TS reeds gepoogd is om de vruchtbaarheid te sparen middels OTC of het invriezen van rijpe eicellen (OC). Helaas is de effectiviteit van deze technieken voor deze specifieke patiëntengroep vooralsnog onbekend door een gebrek aan follow-up data.

Bij jonge kankerpatiënten is OTC al wel een gevestigde methode om de vruchtbaarheid te sparen. Autotransplantatie van gecryopreserveerd ovariumweefsel bij overlevenden van kanker heeft geresulteerd in het herstel van de ovariële functie, zwangerschappen en meer dan 130 levendgeborenen. De kans op het herstel van de ovariële functie is grotendeels gerelateerd aan het aantal follikels in het autotransplantaat. Idealiter worden daarom de ovariumfragmenten met het hoogste aantal follikels geselecteerd voor autotransplantatie. Dit is van klinisch belang voor alle patiënten die autotransplantatie van gecryopreserveerd ovariumweefsel ondergaan, maar vooral voor patiënten die bij OTC al over een verminderde ovariële reserve beschikken zoals patiënten met TS. Het bepalen van de folliculaire dichtheid met conventionele histologie maakt het weefsel echter ongeschikt voor autotransplantatie. Bovendien zijn de follikels niet gelijkmatig verdeeld over het oppervlak van de ovariële cortex, en daarom geeft het bepalen van de follikeldichtheid in één weefselfragment niet noodzakelijkerwijs het aantal follikels in de andere fragmenten weer. Idealiter wordt daarom gebruik gemaakt van een niet-invasieve beeldvormende techniek om het aantal follikels in elk ovariumcortexfragment te bepalen, zodat de fragmenten met de hoogste follikeldichtheid als eerste geselecteerd kunnen worden voor autotransplantatie. In **Hoofdstuk 3** hebben we onderzocht of *reflectance confocal microscopy* (RCM) een geschikte niet-invasieve beeldvormende techniek is om het aantal follikels, en de verticale en horizontale verdeling, in humaan ovariumweefsel te bepalen. In deze proof of concept-studie laten we zien dat RCM een veelbelovende techniek is om de dichtheid van follikels *ex vivo* in ovariumweefsel te bepalen zonder de vitaliteit van het weefsel in gevaar te brengen. Veiligheidsstudies en verdere optimalisatie van de RCM-techniek, met name het vergroten van de penetratiediepte in ovariumweefsel, zijn vereist voordat deze techniek in de routinezorg kan worden toegepast.

Het uitbreiden van de doelgroep voor vruchtbaarheidssparende technieken zoals OTC met meisjes en jonge vrouwen met TS is een ingewikkeld medisch en ethisch vraagstuk en blijft daarom een controversieel onderwerp voor veel clinici. Patiëntenorganisaties

zijn echter optimistisch en eisen een gelijke toegang tot vruchtbaarheidssparende technieken wereldwijd. In **Hoofdstuk 4** onderzochten we de opinie van een internationaal expert panel, bestaande uit zowel professionals als patiëntenvertegenwoordigers, of OTC al dan niet moet worden aangeboden aan meisjes en jonge vrouwen met TS. Tijdens dit Delphi-onderzoek werden eerst de voor- en nadelen van OTC bij meisjes en jonge vrouwen met TS systematisch in 3 rondes bediscussieerd. Het doel hiervan was om panel consensus te bereiken en een internationaal aanvaard standpunt te vormen met betrekking tot OTC bij meisjes en jonge vrouwen met TS. Ons expertpanel concludeerde vervolgens dat OTC moet worden aangeboden aan patiënten met TS, maar alleen in een veilige en gecontroleerde onderzoekssetting. Deze goedkeuring is de eerste stap naar de wereldwijde acceptatie van OTC bij meisjes en jonge vrouwen met TS.

Een gerandomiseerde gecontroleerde studie (RCT) wordt beschouwd als de beste onderzoeksstrategie voor het testen van de effectiviteit van een nieuwe behandeling. Echter, is het, naar onze mening, niet alleen onethisch maar ook logistiek onmogelijk om een laparoscopische ingreep gevolgd door experimentele OTC uit te voeren in een gerandomiseerde gecontroleerde onderzoekssetting (inclusief blindering en behandelingstoewijzing) bij een minderjarige. In **Hoofdstuk 5** stellen we daarom voor om een pilotstudie uit te voeren in plaats van een RCT. We stellen een observationele interventiestudie voor met langdurige follow-up in een tertiaire vruchtbaarheidskliniek in Nederland waarbij een cohort van 100 vrouwen van 2 tot 18 jaar met klassieke Turner (dwz 45, X monosomie) of Turner-varianten (dwz 45, X-mozaïcisme of structurele anomalieën, behoudens de aanwezigheid van Y-chromosomaal materiaal) zal worden geïncludeerd. Het doel van deze studie is om te kijken of OTC op jonge leeftijd, gevolgd door autotransplantatie op volwassen leeftijd, bij vrouwen met TS ook zal leiden tot levendgeboren kinderen. Daarnaast kunnen de studieresultaten gebruikt worden om prognostische markers te vinden voor de aan- of afwezigheid van follikels, zodat de ovariële reserve bij meisjes met TS in de toekomst wellicht beter, en/of al op jonge leeftijd kan worden ingeschat.

Als er follikels worden gevonden in het ovariumweefsel van een meisje of jonge vrouw met TS, rijst vervolgens de vraag of deze follikels ook daadwerkelijk functioneel zijn, dat wil zeggen of ze ook tot zwangerschappen en (gezonde) nakomelingen zullen leiden. Omdat de weefselfunctionaliteit bij patiënten met TS voor een groot deel afhankelijk lijkt te zijn van het karyotype van de cellen, hebben we een methode ontwikkeld (**Hoofdstuk 6**) om het X-chromosomale gehalte van oöcyten en twee ondersteunende celtypen in het ovarium (granulosacellen en stromacellen) te analyseren met behulp van Fluorescentie in situ hybridisatie (FISH). Vervolgens hebben we een pilotstudie uitgevoerd met ovariële cortexfragmenten afkomstig van tien jonge meisjes met TS.

Bij de vijf patiënten die gediagnosticeerd waren met 45,X mozaïcisme konden er in totaal 46 eicellen geanalyseerd worden, hiervan hadden 42 eicellen (91%) een normaal X-chromosomaal gehalte. Verrassend genoeg waren de omliggende granulosacellen grotendeels 45, X, maar vertoonden de andere granulosacellen grote verschillen in X-chromosoommozaïcisme tussen patiënten maar ook tussen follikels van dezelfde patiënt. Het karyotype van de stromacellen bestond uit verschillende cellijnen (45, X, 46, XX en 47, XXX) en was enigszins vergelijkbaar met het karyotype van de verkregen cellen uit urine, lymfocyten en/of buccale cellen. Deze resultaten laten zien dat de mate van X-chromosoommozaïcisme bij patiënten met TS sterk varieert, niet alleen tussen patiënten maar ook binnen de verschillende weefsels en celtypes van een dezelfde patiënt. Bij de vijf patiënten met klassieke TS werden geen follikels gevonden en de karyotypering van de ovariële stromacellen vertoonde uitsluitend 45, X-cellen.

De kans dat er bij een 45, X monosomiepatiënt follikels worden gevonden, is volgens eerder onderzoek ook relatief klein. Sommige clinici stellen daarom voor om OTC alleen beschikbaar te maken voor vrouwen met mozaïek TS. Op dit moment denken we dat het te voorbarig is om de deuren al voor bepaalde subgroepen te sluiten, puur gebaseerd op het karyotype van 20-30 lymfocyten. Om dit te illustreren, beschrijven we een casus (**Hoofdstuk 7**) van cryptisch mozaïcisme bij een 13-jarige meisje bij wie op 8-jarige leeftijd de diagnose 45, X-monosomie of klassieke TS was gesteld. Desondanks trad er een spontane puberteitsontwikkeling op en onderging zij recentelijk een laparoscopische operatie ten behoeve van OTC. Ovariumweefsel kon met succes worden gecryopreserveerd en er werden daadwerkelijk follikels gevonden. Alle primordiale follikels die werden onderzocht, bevatten oocyten met normaal tetraploïde 92, XXXX karyotype, maar waren uitsluitend omgeven door diploïde 45, X granulosacellen. Karyotypering van de stromacellen vertoonde een mozaïekpatroon met 3 cellijnen 45, X / 47, XXX / 46, XX, wat wijst op de aanwezigheid van extra cellijnen naast 45, X.

Concluderend is OTC een veelbelovende methode om de vruchtbaarheid bij vrouwen met TS te sparen. De effectiviteit van OTC bij meisjes en jonge vrouwen met TS is momenteel echter hypothetisch. Daarom stellen we voor dat OTC bij patiënten met TS uitsluitend wordt aangeboden in een veilige en gecontroleerde onderzoeksomgeving. De TurnerFertility trial (CCMO NL57738.000.16, ClinicalTrial ID NCT03381300) is voortgekomen uit de voorbereidingen die in dit proefschrift zijn getroffen.

Appendix

Abbreviations
Research data management
PhD portfolio
Bibliography
Curriculum Vitae
Acknowledgements

List of abbreviations

AMH	Anti-Müllerian Hormone
ART	Assisted Reproductive Technology
BMI	Body Mass Index
BROK	Basiscursus Regelgeving En Organisatie Voor Klinisch Onderzoekers
CCMO	Centrale Commissie Mensgebonden Onderzoek
CE	Conformité Européenne
CEP	Centromere Specific Probe
CV	Curriculum Vitae
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DSD	Disorders Of Sexual Differentiation
DSMB	Data Safety Monitoring Board
E2	Estradiol
EDC	Electronic Data Capture
EDTA	Ethylenediaminetetraacetic Acid
Et al.	Et Alii, And Others
FBS or FCS	Foetal Bovine Serum Or Foetal Calf Serum
FFPE	Formalin Fixated And Paraffin Embedded
FISH	Fluorescence *In Situ* Hybridization
FP	Fertility Preservation
FSH	Follicle-Stimulating Hormone
GCP	Good Clinical Practice
HE	Haematoxylin And Eosin Stain
HIV	Human Immuno-Deficiency Virus
IBM	International Business Machines Corporation
IC	Informed Consent
IQR	Interquartile Range
ISO	International Organization For Standardization
IVA	In Vitro Activation
IVM	In Vitro Maturation
IVF	In Vitro Fertilisation
KCL	Potassium Chloride
LBR	Live Birth Rate
LH	Luteinizing Hormone
METC	Medisch Ethische Toetsing Commissie

OC	Cryopreservation Of Mature Oocytes
OCT	Optical Coherence Tomography
OTC	Ovarian Tissue Cryopreservation
PhD	Doctor (Of Philosophy), Academic Title
POI	Premature Ovarian Insufficiency
PRISMA-P	Preferred Reporting Items For Systematic Review And Meta-Analysis Protocols
RAND / UCLA	Research And Development / University Of California Los Angeles
RCM	Reflectance Confocal Microscopy
(S)AE	(Serious) Adverse Event
SD	Standard Deviation
SPSS	Statistical Package For The Social Sciences
SUSAR	Suspected Unexpected Serious Adverse Reaction
TS	Turner Syndrome
TCN	Turner Contact Nederland
VWS	Ministerie Van Volksgezondheid, Welzijn En Sport
WMA	World Medical Association
WHO	World Health Organization
WMO	Wet Medisch-Wetenschappelijk Onderzoek Met Mensen

Research data management

This thesis is based on the results of human studies, which were conducted in accordance with the principles of the Declaration of Helsinki, the Medical Research Involving Human Subjects Act (WMO), the Guideline for Good Clinical Practice, and all other applicable regulatory requirements.
For Chapter 2 and 4, no ethical review board approval was obtained, as these studies did not involve human subjects or tissue. The study conducted in Chapter 3 was approved by three local ethical committees for patient-related research, i.e. the Ethical committee Arnhem and Nijmegen, reference numbers: 2016-2894 and 2016-2871, date of approval: 12 October 2016 and 19 December 2016; the Ethical committee Amsterdam, reference number: 2017.168, date of approval: 28 March 2017; and the Ethical committee Bonn reference number: 007/09, date of approval: 11 May 2008. Chapter 5, 6 and 7 are part of the TurnerFertility trial, for which ethical approval was obtained by the Dutch Central Committee on Research Involving Human Subjects in 2017 (CCMO NL57738.000.16).
All biological material and data is handled and stored according to the World Medical Association (WMA) Declaration of Taipei on ethical considerations regarding health databases and biobanks (67th WMA General Assembly. Taipei, Taiwan. October 2016). All patient data is coded. Members of the research team, the Data and Safety Monitoring Board, and the Health Care Inspectorate are the only persons who have access to the key of the code. This key of code is stored in Castor Electronic Data capture (EDC).
A safe storage of the ovarian tissue and consent forms is provided at our cryobank. This cryobank is ISO-accredited (accreditation number M101, ISO 15189), and registered by the Dutch Ministry of Health, Welfare and Sport (VWS) (registration number 5515 L/EO). The cryobank is located at a restricted area in the Radboud University Medical Centre, and access is permitted by electronic authorization only. All patients and/or their parents had provided written informed consent.
Research data and study scripts are stored on the Radboudumc department server (H:)\\\Umcfs049\verlgyndata$\Onderzoek\VPG- FP and Turner. Published data generated of analysed in this thesis are parts of published articles. All published articles in this thesis are saved in the Radboud Repository. Chapter 5 and 6 are published open access. Additional files are available from the corresponding author on request.

PhD portfolio

Institute for Health Sciences
Radboudumc

Name PhD candidate:
M.J. Schleedoorn
Department:
Obstetrics and Gynaecology
Graduate School:
Radboud Institute for Health Sciences

PhD period:
01-03-2016 / 31-08-2020
Promotor(s):
Prof. dr. D.D.M. Braat
Co-promotor(s):
dr. K. Fleischer, dr. A.A.E.M. v/d Velden, dr. R. Peek

		Year(s)	ECTS
	TRAINING ACTIVITIES		
a)	**Courses & Workshops**		
-	Introduction day Radboudumc	2016	0.5
-	Endnote workshop	2016	0.1
-	Pubmed introduction course	2016	0.1
-	RIHS introductory course	2016	1.0
-	BROK course	2016	1.5
-	CaRe course on qualitative research	2016	1.0
-	Scientific Integrity course	2017	1.0
-	DSMB course	2018	0.5
-	Safety Training for Liquid Nitrogen	2018	0.1
-	Art of presenting science	2018-2019	2.0
-	Scientific Writing	2019	4.0
-	ESHRE pre-congress course Fertility Preservation	2019	0.5
-	Endocrinology course (Utrecht, NL)	2019	0.5
b)	**Seminars & lectures**		
-	Boerhaave Fertility Preservation (Leiden, NL) (oral presentation)	2018	0.5
c)	**Symposia & congresses**		
-	Conference on Shared decision making (panel member)	2016	0.1
-	Symposium on Cryopreservation (Elsendorp, NL)	2017	0.1
-	Symposium of the Dutch-Belgian Reproductive Research Society (Utrecht, NL)	2016	0.1
-	Symposium of the Dutch-Belgian Reproductive Research Society (Tilburg, NL) (oral presentation)	2018	0.5
-	Symposia for Fertility doctors (Amersfoort, NL)	2016-2019	0.3
-	Petit pain pensant on sexual diversity (Nijmegen, NL)	2017	0.1
-	DSD symposium (Nijmegen, NL)	2019	0.1
-	ESHRE congress (Geneva, CH) (poster discussion and poster presentation)	2017	0.5
-	ESHRE congress (Vienna, A) (oral presentation)	2019	0.5
-	Gynaecongress (Amersfoort, NL) (oral presentation)	2018	0.5
-	Resident 'Refereeravond' (Nijmegen, NL) (oral presentation)	2018	0.5
-	Endowest meeting (Bochum, DE) (oral presentation)	2019	0.5

d)	**Other**		
-	Journal clubs/Scientific meetings Reproductive Medicine	2016-2020	2.0
-	Guideline development member of the Dutch-Belgian guideline on Turner Syndrome care, chapter 'Fertility and pregnancy'	2017-2020	1.0
-	ESHRE young ambassador		
-	Peer reviewing medical papers related to Turner syndrome and fertility preservation	2017	0.5
-	PhD retreat, session chair	2016-2021	0.2
-	Participation in focus group 'Evaluation and improvement of fertility preservation care in females with Turner syndrome'	2018 2019	0.1 0.2
	TEACHING ACTIVITIES		
e)	**Lecturing**		
-	Lecture for gynaecologists, gynaecology residents and medical students	2016-2020	0.1
-	Lecture for paediatricians, paediatrics residents and medical students	2016-2020	0.1
f)	**Supervision of internships / other**		
-	Supervision of 3 Bachelor students (2nd year of Medicine)	2016-2017	3.0
-	Supervision of 3 student-assistants	2017-2019	2.0
-	Supervision of 2 Master students for research internship (6th year of Medicine)	2018	8.0
-	Mentoring 1 Master student for research internship (6th year of Medicine)	2018-2019	1.0
-	Mentoring 1 Bachelor student for excellent student programme (2nd year of Medicine)	2018-2019	1.0
	TOTAL		**36.3**

^Indicate oral or poster presentation

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Submitted

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Oral presentations

European Society of Human Reproduction and Embryology - International consensus: Ovarian tissue cryopreservation in young Turner syndrome patients, 2019, Vienna, Austria

Endowest symposium - Ovarian Tissue cryopreservation in young females with Turner Syndrome: preliminary results, 2019, Bochum, Germany

Resident Research 'Refereeravond' - 'Het invriezen van eierstokweefsel bij meisjes met het syndroom van Turner: een ethisch vraagstuk', 2018, Nijmegen, The Netherlands

Gynaecongres - 'To freeze or not to freeze? Ethische aspecten die komen kijken bij het invriezen van eierstokweefsel van meisjes met het syndroom van Turner', 2018, Amersfoort, The Netherlands

Boerhaave symposium Bevroren Geluk - 'Fertiliteitspreservatie bij het syndroom van Turner', 2018, Leiden, The Netherlands

The Dutch-Belgian Reproductive Research Society: 'Patiëntparticipatie bij medisch wetenschappelijk onderzoek – over de TurnerFertility trial', 2018, Tilburg, The Netherlands

Poster presentations

European Society of Human Reproduction and Embryology (poster discussion): Ethical aspects of ovarian tissue freezing in young females with Turner Syndrome, 2017, Genève, Switzerland

European Society of Human Reproduction and Embryology (poster): Non-invasive imaging of living ovarian tissue with reflectance confocal microscopy, 2017, Genève, Switzerland

Curriculum Vitae

Myra Schleedoorn werd op 5 maart 1989 geboren in het Zuwe Hofpoort Ziekenhuis als oudste in een gezin van twee kinderen. Zij groeide op in Woerden, Maarssen en Elst (Utr.) en behaalde haar Gymnasiumdiploma aan het Christelijk Lyceum te Veenendaal. Na kort getwijfeld te hebben over de Kunstacademie, besloot zij Geneeskunde te studeren aan de Radboud Universiteit te Nijmegen. Tijdens haar studie bleef zij breed geïnteresseerd en was actief bij verschillende organisaties die zich inzetten voor mens en dier.

Tijdens haar coschap Gynaecologie en Verloskunde in het Maasziekenhuis Pantein te Boxmeer (2014) ontdekte zij hoe mooi en veelzijdig het vak gynaecologie is. Met name de begeleiding van kwetsbare zwangeren en de ethische vraagstukken rondom geassisteerde voortplanting trokken haar interesse. Dit gevoel werd bevestigd tijdens haar seniorcoschap in het Canisius Wilhelmina Ziekenhuis te Nijmegen (2015) en keuzecoschappen in het Bernhoven Ziekenhuis te Uden (2015).

Daarnaast lonkte de wetenschap. Na eerst ervaring op te hebben gedaan als student-assistent op het FertiScreen project (2014-2015), mocht zij in 2015 haar 'eigen' onderzoek uitvoeren onder begeleiding van dr. Nelen (Radboudumc) en dr. Dunselman (MUMC+). Dit resulteerde in twee publicaties.

In februari 2016 nam zij haar artsenbul in ontvangst en kon direct aan de slag als fertiliteitsarts in het Radboudumc te Nijmegen. Zij combineerde deze baan met een promotietraject onder leiding van prof. dr. Braat, dr. Nelen, dr. Fleischer en dr. van der Velden. Tijdens het traject nam dr. Peek het 'copromotor' stokje van dr. Nelen over. Dit resulteerde in een multidisciplinair proefschrift met zowel kwantitatieve- als kwalitatieve onderzoeksmethoden.

Myra is sinds 2009 samen met Alexander. Eind 2019 werd hun liefde bekroond met de geboorte van Floyd. Begin 2021 is zij met haar gezin geëmigreerd naar Lohr am Main (Beieren) waar zij samen met haar partner een oud vakwerkhuis renoveert en zij zich verder zal specialiseren tot vrouwenarts.

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